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SWEET17, a facilitative transporter, mediates fructose transport across the tonoplast of Arabidopsis roots and leaves

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Abstract: Fructose (Fru) is a major storage form of sugars found in vacuoles, yet the molecular regulation of vacuolar Fru transport is poorly studied. Although SWEET17 (for SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS17) has been characterized as a vacuolar Fru exporter in leaves, its expression in leaves is low. Here, RNA analysis and SWEET17-*-glucuronidase/-GREEN FLUORESCENT PROTEIN* fusions expressed in Arabidopsis (*Arabidopsis thaliana*) reveal that SWEET17 is highly expressed in the cortex of roots and localizes to the tonoplast of root cells. Expression of SWEET17 in roots was inducible by Fru and darkness, treatments that activate accumulation and release of vacuolar Fru, respectively. Mutation and ectopic expression of SWEET17 led to increased and decreased root growth in the presence of Fru, respectively. Overexpression of SWEET17 specifically reduced the Fru content in leaves by 80% during cold stress. These results intimate that SWEET17 functions as a Fru-specific uniporter on the root tonoplast. Vacuoles overexpressing SWEET17 showed increased [¹⁴C]Fru uptake compared with the wild type. SWEET17-mediated Fru uptake was insensitive to ATP or treatment with NH₄Cl or carbonyl cyanide *m*-chlorophenyl hydrazone, indicating that SWEET17 functions as an energy-independent facilitative carrier. The Arabidopsis genome contains a close paralog of SWEET17 in clade IV, SWEET16. The predominant expression of SWEET16 in root vacuoles and reduced root growth of mutants under Fru excess indicate that SWEET16 also functions as a vacuolar transporter in roots. We propose that in addition to a role in leaves, SWEET17 plays a key role in facilitating bidirectional Fru transport across the tonoplast of roots in response to metabolic demand to maintain cytosolic Fru homeostasis.

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1 **Running Title:**

2 SWEET17 transports fructose across the root tonoplast

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3 **SWEET17, a facilitative transporter, mediates fructose transport across the**
4 **tonoplast of Arabidopsis roots and leaves**

5

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14 vacuole

15

Abstract

Fructose (Frc) is a major storage form of sugars found in vacuoles, yet the molecular regulation of vacuolar Frc transport is poorly studied. Although *SWEET17* has been characterized as a vacuolar Frc exporter in leaves, its expression in leaves is low. Here, RNA analysis and *SWEET17- β -glucuronidase/-green fluorescent protein* fusions expressed in *Arabidopsis* revealed that *SWEET17* was highly expressed in the cortex of roots and showed clear tonoplast localization in root cells. Expression of *SWEET17* in roots was induced by Frc supply and darkness that activate accumulation and release of vacuolar Frc, respectively. Mutation and ectopic expression of *SWEET17* led to increased and decreased root growth in the presence of Frc, respectively. Overexpression of *SWEET17* specifically reduced the Frc content in leaves by 80% during cold stress. These results intimate that *SWEET17* functions as a Frc-specific uniporter on the root tonoplast. Indeed, vacuoles overexpressing *SWEET17* showed an enhanced uptake rate to ^{14}C -labeled Frc compared to the wildtype. *SWEET17*-mediated Frc uptake was insensitive to ATP and treatments with NH_4Cl or CCCP, supporting that *SWEET17* function as an energy-independent facilitative carrier. The *Arabidopsis* genome contains a close paralog of *SWEET17* in clade IV, *SWEET16*. The predominant expression of *SWEET16* in root vacuoles and reduced root growth of mutants under Frc excess indicate that *SWEET16* also functions as a vacuolar transporter in roots. We propose that beside a role in leaves, *SWEET17* plays a key role in facilitating bi-directional Frc transport across the tonoplast of roots upon metabolic demand to maintain cytosolic Frc homeostasis.

1 Sugars are the main energy sources to generate adenosine triphosphate, the major
2 precursors to various storage carbohydrates as well as key signaling molecules important
3 for normal growth in higher plants (Rolland et al., 2006). Depending on metabolic
4 demand, sugars are transported over a long distance or stored intracellularly. SWEET and
5 SUT-type sucrose (Suc) transporters are responsible for cellular efflux and load Suc from
6 the phloem parenchyma into the sieve element companion cell complex for long distance
7 translocation (Riesmeier et al., 1992; Sauer, 2007; Kühn and Grof, 2010; Chen et al.,
8 2012). Suc or hexoses derived from Suc hydrolysis in the cell wall are then taken up into
9 sink cells by SUT transporters (Braun and Slewinski, 2009) or monosaccharide
10 transporters (Pego and Smeekens, 2000; Sherson et al., 2003), such as sugar transporter 1,
11 AtSTP1 (Sauer et al., 1990). Alternatively, sugars can move between cells via
12 plasmodesmata (Voitsekhovskaja et al., 2006; Ayre, 2011). The major sugar storage
13 pools within a plant cell are the plastid, where starch is either stored transiently or as a
14 long term reserve, and the vacuole.

15 Vacuoles, which account for 80 to 90% of the cell volume (Winter et al., 1993), play
16 central roles in temporary and long-term storage of soluble sugars (Martinoia et al., 2007;
17 Etxeberria et al., 2012). Some agriculturally important crops like sugar beet (Leigh, 1984;
18 Getz and Klein, 1995), citrus (Echeverria and Valich, 1988), sugar cane (Thom et al.,
19 1982) and carrot (Keller, 1988) can store considerable amounts (> 10% of plant dry
20 weight) of Suc, Glucose (Glc) or Frc in vacuoles of the storage parenchyma. Due to a
21 high capacity of vacuoles for storing sugars, vacuolar sugars can serve as the important
22 carbohydrate supply during energy starvation after starch has been exhausted (Echeverria
23 and Valich, 1988) as well as for the production of other compounds (e.g.
24 osmoprotectants). Sugars are known to regulate photosynthesis; therefore the release of
25 sugars from vacuoles could be important for modulating photosynthesis (Kaiser and
26 Heber, 1984). Moreover, vacuole-derived sugars are commercially used to produce
27 biofuels, such as ethanol, from sugarcane. Knowledge of the key transporters involved in
28 sugar exchange between the vacuole and cytoplasm is thus relevant in the context of
29 bioenergy (Grennan and Gragg, 2009).

30 To facilitate the exchange of sugars across the tonoplast, plant vacuoles are
31 equipped with a multitude of transporters (Neuhaus, 2007; Etxeberria et al., 2012;

1 Martinoia et. al., 2012) comprising both facilitated diffusion and active transport systems
2 of vacuolar sugars (Martinoia et al., 2000). Typically, Suc is actively imported into
3 vacuoles by the tonoplast monosaccharide transporter (AtTMT1/2) (Schulz et al., 2011)
4 and exported by the SUT4 family (AtSUC4, OsSUT2) (Eom et al., 2011; Payyavula et al.,
5 2011; Schulz et al., 2011). Two H⁺-dependent sugar antiporters, the vacuolar Glc
6 transporter (AtVGT1) (Aluri and Buttner, 2007) and AtTMT1 (Wormit et al., 2006),
7 mediate Glc uptake across the tonoplast to promote carbohydrate accumulation in
8 Arabidopsis. The Early Responsive to Dehydration-Like 6 (AtERDL6) protein has been
9 shown to export vacuolar Glc into the cytosol (Poschet et al., 2011), likely via an energy-
10 independent diffusion mechanism (Yamada et al., 2010). Defects in these vacuolar sugar
11 transporters alter carbohydrate allocation and inhibit plant growth and seed yield (Aluri
12 and Buttner, 2007; Wingenter et al., 2010; Eom et al., 2011; Poschet et al., 2011).

13 In contrast to numerous studies on vacuolar transport of Suc and Glc, limited efforts
14 had been devoted to the molecular mechanism of vacuolar Frc transport even though Frc
15 is predominantly located in vacuoles (Martinoia et al., 1987; Voitsekhovskaja et al., 2006;
16 Tohge et al., 2011). Vacuolar Frc is important in turgor pressure regulation (Pontis, 1989),
17 anti-oxidative defense (Bogdanovi et al., 2008), and signal transduction during early
18 seedling development (Cho and Yoo, 2011; Li et al., 2011). Thus, control over Frc
19 transport across the tonoplast is thought to be important for plant growth and
20 development. One vacuolar Glc transporter from the Arabidopsis MST family, AtVGT1,
21 has been reported to mediate low affinity Frc uptake when expressed in yeast vacuoles
22 (Aluri and Buttner, 2007). Yet, the high vacuolar uptake activity to Frc intimates the
23 existence of additional high capacity Frc-specific vacuolar transporters (Thom et al.,
24 1982). Recently, quantitative mapping of a quantitative trait locus for Frc content led to
25 the identification of a Frc-specific vacuolar transporter, SWEET17 (Chardon et al., 2013).

26 SWEET17 belongs to the recently identified SWEET (PFAM:PF03083) super
27 family, which contains 17 members in Arabidopsis and about 21 homologs in rice (Chen
28 et al., 2010; Frommer et al., 2013; Xuan et al., 2013). Based on amino acid similarity,
29 plant SWEET proteins were grouped into four subclades exhibiting 27 to 80% identity
30 (Chen et al., 2010). Transport assays using radiotracers in *Xenopus* oocytes or sugar
31 nanosensors in mammalian cells showed that most SWEET transporters are plasma

1 membrane localized and function as low-affinity and pH-independent uniporters that
2 have both uptake and efflux activity (Chen et al., 2010; Chen et al., 2012). In particular,
3 clade I and II SWEETs transport monosaccharides and clade III SWEETs transport
4 disaccharides, mainly Suc (Chen et al., 2010; Chen et al., 2012). Mutant phenotypes and
5 developmental expression of several SWEET transporters support important roles in
6 sugar allocation between organs. The clade III SWEETs, SWEET11 and 12 mediate the
7 key step of Suc efflux from phloem parenchyma cells for phloem translocation (Chen et
8 al., 2012). SWEETs are co-opted by pathogens to provide energy resources and carbon at
9 the site of infection (Chen et al., 2010). Mutations of *SWEET8/AtRPG1* in Arabidopsis,
10 *OsSWEET11* (also called *Os8N3* or *Xa13*) in rice, and petunia *NECI* resulted in male
11 sterility (Ge et al., 2001; Yang et al., 2006; Guan et al., 2008), possibly caused by
12 inhibiting the Glc or Suc transport required for pollen or pollen tube function (Guan et
13 al., 2008). Similarly, mutations of *SWEET17* expression caused Frc accumulation in
14 Arabidopsis leaves, indicating that it plays a key role in exporting Frc from leaf vacuoles
15 (Chardon et al., 2013). A more recent study has demonstrated that SWEET16 also
16 functions as a vacuolar sugar transporter using the same heterologous system as shown
17 for SWEET17 (Klemens et al., 2013). Surprisingly, however, *SWEET17* expression in
18 mature leaves was comparatively low (Chardon et al., 2013), which leads us to ask the
19 question whether this transporter could play a major role in another tissue or under
20 specific developmental or environmental conditions. Although Arabidopsis SWEET17
21 has been shown to transport Frc in a heterologous system where accumulated in part at
22 the plasma membrane (Chardon et al., 2013), the biochemical properties of SWEET17
23 transport *in vivo* were still elusive. SWEET17 and 16 from Arabidopsis belong to the
24 clade IV SWEETs. Whether clade IV proteins both transport vacuolar sugars *in planta*
25 deserves further studies.

26 Here, we used GUS/GFP fusions to reveal the root-dominant expression and
27 vacuolar localization of the SWEET17 protein *in vivo* and its regulation in response to
28 intracellular Frc. Phenotypes of mutants and overexpressors were consistent with a role
29 of SWEET17 in bi-directional Frc transport across root vacuoles. The uniport feature of
30 SWEET17 transport *in vivo* was further confirmed using isolated mesophyll vacuoles.
31 Using the same approaches, SWEET16 was also shown to function in vacuolar sugar

1 transport in roots. Our work, performed in parallel, provide direct evidences to show the
2 Frc-specific uniport activity of SWEET17 *in planta* proposed by a recent study (Chardon
3 et al., 2013) and presents functional analyses to uncover important roles of these vacuolar
4 transporters in maintain intracellular Frc homeostasis in sink cells, roots.

5 **Results**

6 **SWEET17 proteins are highly expressed in roots**

7 A very recent report had indicated that SWEET17 (At4g15920) functions as a Frc
8 exporter in leaf vacuoles. However, *SWEET17* expression appeared to be very low in
9 leaves (Chardon et al., 2013), indicating that SWEET17 may predominantly function in
10 other **sink organs than leaves** under specific environmental conditions. A quantitative
11 reverse transcription (qRT)-PCR analysis revealed that *SWEET17* mRNA was expressed
12 to high levels in roots of 2-week-old seedlings (Fig. 1). In soil-grown mature plants, some
13 aerial organs, i.e. stems, flowers and siliques also accumulated high levels of *SWEET17*
14 transcripts. By contrast, expression of *SWEET17* was comparatively low in both young
15 and mature leaves (Fig. 1). The high levels of *SWEET17* transcripts in roots observed
16 here correlated well with the steady state expression profile from the Arabidopsis eFP
17 Browser (Supplemental Fig. S1A) (Winter et al., 2007) and the Translatome database
18 **(polysome-bound mRNA)** (Mustroph et al., 2009) (Supplemental Fig. S1B). Since steady
19 state mRNA levels do not necessarily reflect protein abundance (Krügel and Kühn, 2013),
20 translational fusions were analyzed. We generated transgenic Arabidopsis plants
21 expressing a C-terminal translational GUS gene fusion of SWEET17 driven by the native
22 *SWEET17* promoter (SWEET17-GUS). In particular, the full length of *SWEET17* gene
23 containing all introns was used to observe the genuine expression of the protein *in planta*.
24 In 7-d-old transgenic seedlings, SWEET17-GUS fusion proteins were mainly found in
25 cotyledons and roots (Fig. 2A). A similar expression pattern was also observed in 2-
26 week-old seedlings (Fig. 2B), where, however, much lower GUS staining was seen in
27 aerial tissues. The expression pattern of SWEET17 proteins was also consistent with the
28 expression pattern analyzed by a GUS reporter driven by the *SWEET17* promoter
29 (Supplemental Fig. S1C). In roots, SWEET17 was predominantly expressed in root tips
30 (Fig. 2C) and mature regions of roots (Fig. 2D). Only low expression was observed in the

1 elongation zone of roots (Fig. 2C). Three independent reporter lines showed comparable
2 patterns of GUS staining (data not shown). Handsections of mature roots histochemically
3 stained for GUS activity further demonstrated that SWEET17 predominantly
4 accumulated in the root cortex (Fig. 2E). The cell-type specific expression was
5 comparable with that of root array data from the Arabidopsis eFP Browser (Supplemental
6 Fig. S2, A and B) and the Translatome database (Supplemental Fig. S2C). In soil-grown
7 mature plants, expression of SWEET17-GUS was consistently observed to be high in
8 roots and low in aerial tissues, such as leaves, stems and flowers (Supplemental Fig.
9 S3A). When the reaction time of GUS staining was doubled (to 4 h), low and patchy
10 expression of the SWEET17-GUS fusion proteins was observed in mature leaves (Fig.
11 2F). After extended staining, some GUS activity was also observed in the vascular tissues
12 of flowers (Fig. 2G) and the bottom of siliques (Fig. 2H), but not in seeds (Fig. 2I).

13 **Expression of SWEET17 is regulated by Frc in roots**

14 Since expression of some sugar transporters is modulated by altering sugar contents
15 (Williams et al., 2000), we investigated if altered sugar levels would affect the spatial
16 pattern of expression of SWEET17. Transgenic Arabidopsis seedlings expressing
17 SWEET17-GUS fusion proteins were grown on media supplemented with 1% Suc for 5 d
18 then transferred to media without or with 1% Suc, Glc, or Frc for 2 d. Histochemical
19 staining for GUS activity showed that SWEET17 accumulation was similar in the
20 presence of Suc or Glc (Supplemental Fig. S3B), but highly induced by 1% Frc in
21 elongation regions of roots compared to control conditions (Fig. 2, J and K). To address
22 whether SWEET17 expression responds to a low intracellular sugar status, seedlings
23 expressing SWEET17-GUS fusion proteins were grown on media supplemented with 1%
24 Suc under 16 h daylength for 5 d then transferred to sugar-free media in the dark for
25 additional 2 d. The extended dark period leads to sugar starvation in plant cells (Usadel et
26 al., 2008). Interestingly, accumulation of SWEET17-GUS fusion proteins in elongation
27 regions of roots was also highly induced by darkness (Fig. 2, J and L). The same Frc and
28 sugar-starvation inducible patterns in roots were observed at the transcriptional level
29 using a *SWEET17* promoter:GUS fusion (Supplemental Fig. S3C). Weak induction of
30 SWEET17-GUS expression in leaves was also observed upon exposure to darkness or by
31 combining darkness with cold stress (Supplemental Fig. S4). These observations were

1 consistent with a predicted role of SWEET17 as a Frc uniporter for uptake of excess
2 cytosolic Frc or for release the stored Frc across the vacuolar membrane to maintain
3 homeostasis.

4 **SWEET17 proteins in root vacuoles**

5 To investigate whether SWEET17 is targeted to the tonoplast also in roots, we
6 generated transgenic Arabidopsis plants expressing a C-terminal translational GFP fusion
7 of SWEET17 (SWEET17-GFP) driven by the native *SWEET17* promoter. To allow
8 possible transcriptional regulation, the full length genomic *SWEET17* gene was used.
9 Confocal images of intact roots from homozygous transformants revealed that the
10 fluorescence derived from SWEET17-GFP fusion proteins was predominantly present at
11 the tonoplast of root tips (Fig. 3A). The vacuolar localization was evidenced by
12 fluorescence surrounding small pre-mature vacuoles located inside the plasma membrane
13 labeled by FM4-64 (Fig. 3, B and C) (Wayne, 2009). The vacuolar pattern was also
14 observed in mature regions of roots as shown by fluorescence lining the inner side of the
15 nucleus and plasma membrane (Fig. 3, D-F) (Wayne, 2009). The fluorescence derived
16 from SWEET17-GFP fusion proteins in the cytosol or at the plasma membrane was not
17 significant. Tonoplast-specific localization of SWEET17-GFP was also observed in
18 mesophyll protoplasts (Supplemental Fig. S5). Despite overall low levels of fluorescence
19 in leaf tissues, SWEET17-GFP fluorescence in Arabidopsis protoplasts was clearly
20 concaved by chloroplast autofluorescence (Supplemental Fig. S5A) and not detected at
21 the plasma membrane (Supplemental Fig. S5B). These observations indicate that
22 SWEET17 locates to and functions predominantly at the tonoplast of roots.

23 **Import activity of SWEET17 confers tolerance to Frc inhibition**

24 High levels of cytosolic Frc inhibit root growth and arrest seedling development
25 (Bhagyalakshmi et al., 2004; Cho and Yoo, 2011). If SWEET17 could act as a vacuolar
26 importer to store excess Frc in roots, as suggested by the expression pattern (Fig. 2 and 3),
27 altered expression of SWEET17 may affect intracellular Frc allocation and thereby affect
28 sensitivity of roots to Frc. To test this hypothesis, Arabidopsis knockout mutants,
29 *sweet17-1* and *sweet17-2* with T-DNAs inserted in the *SWEET17* gene were obtained
30 (Chardon et al., 2013). While the relative root growth of the segregating wildtype (Col-

TDNA) and mutants (*sweet17-1* and *sweet17-2*) was similar when grown on media with or without 1% Suc, root growth of *sweet17-1* and *sweet17-2* seedlings was significantly more sensitive to 1 to 2% of Frc compared to the wildtype (Fig. 4A). SWEET17 thus appears to be, at least partially, necessary for Frc tolerance. We therefore tested whether SWEET17 is also sufficient for Frc tolerance in roots. We generated transgenic Arabidopsis plants overexpressing the full length genomic *SWEET17* gene driven by the constitutive cauliflower mosaic virus 35S promoter. In 7-d-old seedlings, RT-PCR analysis showed that levels of *SWEET17* transcripts in homozygous transgenic plants were highly increased in overexpressing lines, 35S:*SWEET17*-1, -6 and -2, compared to plants transformed with the empty vector (Col-Vector; Supplemental Fig. S6A). In contrast to the increased sensitivity of root growth in *sweet17* mutants (Fig. 4A), three independent overexpressor lines (35S:*SWEET17*-1, -6 and -2) showed enhanced tolerance compared to wildtype plants (Col-Vector-1 and -2) in the presence of 0.1 to 1% Frc (Fig. 4B). Excess sugars inhibit seed germination (Dekkers et al., 2004). To examine if SWEET17 also contributes to Frc translocation during early seedling development, we compared germination rates of mutants and overexpressors subjected to excess Frc. However, after 2 to 4 d of incubation, we did not observe significant differences between all lines tested (Supplemental Fig. S6, B and C; data not shown).

Overexpressing SWEET17 decreases Frc accumulation in leaves

The induction of SWEET17 expression by energy starvation (Fig. 2, J and L) indicates that SWEET17 may be able not only to import, but also export Frc stored in the vacuole into the cytosol when there is a metabolic demand. To examine how the increased export activity of SWEET17 proteins affects the capacity of vacuoles to store sugars, we determined sugar contents of leaves of SWEET17 overexpressors under standard growth conditions and in response to cold stress (4°C) for 1 week. Cold stress has been shown to induce Frc accumulation in vacuoles by 2 to 10 fold within 24 h (Wormit et al., 2006). Under standard conditions, no dramatic differences in leaf sugar levels were observed when comparing wildtypes and overexpressors (Supplemental Fig. S7A). However, under cold stress conditions, the Frc content of leaves of 35S:*SWEET17*-1 overexpressor plants was significantly reduced by 80% compared to the wildtype transformed with the empty vector (Col-Vector) (Fig. 5). No significant differences in

1 Glc and Suc levels were observed in cold-stressed overexpressors (Fig. 5 and
2 Supplemental Fig. S7B). These results provide functional evidence that SWEET17 is
3 involved in vacuolar Frc export *in planta*.

4 **Direct evidence for a role of SWEET17 as a low affinity tonoplast Frc** 5 **uniporter**

6 The sensitivity of root growth to Frc inhibition in *sweet17* mutants (Fig. 4A) and the
7 decreased Frc content in cold stressed SWEET17 overexpressors (Fig. 5) indicated that
8 SWEET17 mediates bi-directional Frc-specific transport *in vivo*. To directly measure the
9 transport properties of SWEET17, leaf vacuoles were isolated from overexpressors plants
10 grown under standard conditions and used to perform time-course uptake assays with
11 [¹⁴C]Suc, [¹⁴C]Glc, and [¹⁴C]Frc. Vacuoles isolated from 35S:*SWEET17*-1 and -6
12 mesophyll protoplasts exhibited an enhanced time-dependent Frc uptake activity
13 compared to those isolated from control leaves (Col-Vector, Fig. 6A). By comparison, the
14 uptake activities for Glc or Suc were not significantly increased (Supplemental Fig. S8).
15 These results show that SWEET17 can import Frc specifically into vacuoles along a
16 concentration gradient *in vivo*.

17 To confirm if SWEET17 acts as a uniporter as observed from phenotypes (Fig. 4
18 and 5), we examined the effect of ATP and treatments with a uncoupler, NH₄Cl (5 mM)
19 (Wormit et al., 2006) or a protonophore, carbonyl cyanide m-chlorophenyl hydrazone
20 (CCCP, 10 μM) (Eom et al., 2011), on the Frc uptake activity of SWEET17 (Fig. 6B).
21 After an incubation time of 20 min in the presence of 0.2 mM [¹⁴C]Frc and MgATP,
22 vacuoles from plants overexpressing SWEET17 had taken up 2.5 times more Frc uptake
23 than those expressing the empty vector (Fig. 6B). The absence of ATP as well as
24 treatments of NH₄Cl or CCCP, which disrupt the preexisting proton gradient, had no
25 impact on the transport activity (Fig. 6B).

26 To investigate the affinity of the vacuolar Frc transporter, we performed a
27 concentration-dependent uptake experiment. Saturation of Frc uptake was not observed at
28 a concentration even up to 20 mM (Fig. 6C). The deduced K_m and V_{max} values of
29 SWEET17 activity were 25.6 mM and 479.8 pmol Frc μl vacuole⁻¹ min⁻¹, respectively,
30 demonstrating that SWEET17 is a low affinity Frc transporter.

Both clade **IV** SWEETs function as vacuolar transporters in roots

The Arabidopsis genome contains a close homolog of SWEET17 in clade IV, named SWEET16. In a recent study, *SWEET16* (At3g16690) was also shown to function as a vacuolar sugar facilitator in vascular parenchyma cells (Klemens et al., 2013). However, the expression of *SWEET16*, measured as GUS activity driven by the *SWEET16* promoter is relatively low in most tissues, including roots (Klemens et al., 2013). Since SWEET16 and 17 proteins share 70% amino acid identity (Supplemental Fig. S9A), we suspected that SWEET16 may play a partially redundant function with SWEET17 in roots under certain environmental stimuli. To address this hypothesis, we performed similar experiments as for SWEET17. Interestingly, the (qRT)-PCR analysis showed predominant expression of *SWEET16* mRNA in roots compared to all aerial organs, such as leaves, stems and flowers in both young seedlings and mature plants (Fig. 1). The expression pattern was consistent with the developmental expression profile from the Arabidopsis eFP Browser (Supplemental Fig. S1A) and the Translatome database (Supplemental Fig. S1B). To investigate if *SWEET17* and *16* are the only *SWEET* genes highly expressed in roots, we performed (qRT)-PCR to analyze expression levels of all 17 Arabidopsis *SWEET* genes in roots. Under the condition tested, *SWEET17* and *16* genes, which are part of the clade IV (Chen et al., 2010), were the most highly expressed *SWEET* genes; a few other *SWEET* genes were expressed at low levels in roots (Fig. 7). The pattern was consistent with the expression profile derived from the AtGeneExpress database (Supplemental Fig. S9B).

To analyze the tissue-specific expression of SWEET16 proteins, we generated transgenic Arabidopsis expressing C-terminal translational fusions to GUS and GFP under the control of the native *SWEET16* promoter (SWEET16-GUS/GFP), again using the full length *SWEET16* genomic gene. Similar to SWEET17, both SWEET16-GUS fusion proteins and promoter activity were mainly found in roots (Supplemental Fig. S10, A and B). SWEET16 fusions accumulated predominantly in the cortex cells of mature roots (Supplemental Fig. S10, C and D). The cell-type specific expression was consistent with the Arabidopsis eFP Browser (Supplemental Fig. S2, A and B) and the Translatome

1 database (Supplemental Fig. S2C). Confocal images of intact roots in homozygous
2 transformed seedlings clearly demonstrated that the fluorescence of SWEET16-GFP
3 fusion proteins localized to the vacuolar membrane in roots (Supplemental Fig. S10, E-G).
4 To functionally characterize SWEET16 *in planta*, we obtained Arabidopsis *sweet16* T-
5 DNA insertion knockout mutants (Supplemental Fig. S11A) and generated a *sweet17*-
6 *l/sweet16-1* double mutant (Supplemental Fig. S11B) as well as SWEET16
7 overexpressor lines (Supplemental Fig. S11C). Vacuoles isolated from *sweet17*-
8 *l/sweet16-1* showed a markedly decrease in Frc uptake activity (Supplemental Fig.
9 S12A). Although overexpression of *SWEET16* in leaves did not increase the Frc transport
10 activity (Supplemental Fig. S12B), Frc content in leaves was significantly decreased in
11 response to cold stress (Supplemental Fig. S7B) compared to standard growth conditions
12 (Supplemental Fig. S7A). These results imply that SWEET16 is probably involved in Frc
13 transport *in planta*, although at a much lower degree than SWEET17. Consistent with this
14 observation, root growth of *sweet16-1* and *sweet16-2* seedlings was significantly more
15 sensitive to 0.5 to 2% of Frc than the wildtype (Fig. 8A). However, compared to *sweet16*
16 single mutants, the *sweet17-1/sweet16-1* double mutant did not show enhanced sensitivity
17 to excess Frc. In addition, overexpressing SWEET16 significantly enhanced root growth
18 at 0.5 to 2% of Frc (Fig. 8B).

19 Discussion

20 SWEET17 function at the root tonoplast

21 Tight regulation of sugar compartmentation between the cytosol and vacuole is
22 important for proper plant development (Martinoia et al., 2000). A QTL analysis
23 identified SWEET17 as a key player in controlling leaf Frc content and provided
24 evidence that SWEET17 localizes to the vacuole. However, based on promoter-GUS
25 fusions, *SWEET17* expression was restricted to the main vein of the leaf, and was found
26 only in the basal part of the leaf (Chardon et al., 2013). Using (qRT)-PCR analysis and a
27 whole-gene SWEET17-GUS translational fusion, here we show that SWEET17 is highly
28 expressed in roots and confirm that it is only present at low levels in leaves at all
29 vegetative stages (Fig. 1 and 2; Supplemental Fig. S3A). The trend correlated well with
30 results from microarray and translome databases (Supplemental Fig. S1, A and B). In

1 contrast to high levels of steady-state *SWEET17* transcripts detected in stems, flowers and
2 siliques (Fig. 1; Supplemental Fig. S1A), very low levels of SWEET17 proteins analyzed
3 by a GUS fusion were observed in those tissues (Fig. 2F-H; Supplemental Fig. S3A).
4 Organ-specific post-translational regulation may modulate SWEET17 function as been
5 observed for other sugar transporters (Krügel and Kühn, 2013). The discrepancy between
6 our observations using a whole-gene construct and the previous report using a promoter-
7 GUS construct (Chardon et al., 2013) may be due to intragenic regulatory elements
8 within introns or exons that regulate gene expression. That has been observed in the
9 **Arabidopsis** sucrose transporters, AtSUC1 and AtSUC9 (Sivitz et al., 2007) and well
10 described in some nutrient transporters, such as AtAMT1 (Yuan et al., 2007) and
11 AtNRT2 (Laugier et al., 2012). Further studies will be required to discover the regulatory
12 mechanism. Nevertheless, these observations point out that expression of SWEET17 is
13 tightly regulated to cope with the dynamics of cellular sugar homeostasis (Fig. 2, J-L;
14 Supplemental Fig. S3C and S4). The tonoplast-specific localization of SWEET17-GFP
15 in Arabidopsis roots and leaves (Fig. 3; Supplemental Fig. S5) provided a clear evidence
16 of its role on vacuoles as suggested by a transient ectopic expression of SWEET17
17 cDNA-GFP fusion in protoplasts (Chardon et al., 2013). Taken together, these
18 observations point to a potential role of SWEET17 on the root tonoplast.

19 Accumulation of SWEET17 is predominantly found in the cortex cells of mature
20 roots (Fig. 2, D and E; Supplemental Fig. S2, B and C). The cortex cells mainly serve as
21 a storage pool of carbohydrates and metabolites related to biotic and abiotic stresses
22 (Hassan and Mathesius, 2012). In some plant species, the prime location of pathogen or
23 symbiotic bacterial infection in roots occurs through the cortex (Czajkowski et al., 2010).
24 A previous study had shown that OsSWEET11 expressed in rice leaves can be hijacked
25 by pathogens to export sugars for their energy supply during infection (Chen et al., 2010).
26 Several SWEET family members are also highly induced by pathogen infection. Whether
27 SWEET17 is involved in releasing stored vacuolar sugars in root cortex cells during
28 interaction with root microorganisms will be a very interesting aspect to investigate in the
29 future.

30 **SWEET17 regulates Frc homeostasis in roots**

Given that most of Frc is stored in vacuoles (Voitsekhovskaja et al., 2006; Tohge et al., 2011), vacuolar SWEET17 is probably involved in importing excess cytosolic Frc into vacuoles for storage in roots after sugars are unloaded from the phloem stream and hydrolyzed by invertases. Several observations support this notion. In presence of externally supplied Frc, which activates Frc accumulation in vacuoles, expression of SWEET17 was highly induced in the elongation region of roots (Fig. 1, J and K), where active sugar uptake from the outside of roots occurs and most of Frc is accumulated (Jones and Darrah, 1996). Root growth was significantly reduced in *sweet17* mutant plants under excess Frc (Fig. 4A), probably caused by a limited allocation of Frc into vacuoles leading to a higher cytosolic Frc concentration (Cho and Yoo, 2011). The inhibitory effect on root growth could be alleviated by ectopic overexpression of SWEET17 (Fig. 4B). We could also demonstrate vacuolar import activity of SWEET17 *in planta* vacuole transport assays (Fig. 6A). These import features of SWEET17 lead us to conclude that physiologically SWEET17 mediates Frc uptake into vacuoles for storage in response to a high concentration of cytosolic Frc in roots.

Frc stored in root vacuoles may serve as an important source of energy for actively growing cells (Echeverria and Valich, 1988; Etcheberria et al., 2012), such as those in the elongation region where newly formed cells are quickly expanding and accelerate maturation of the organelles. Interestingly, upon 48 h incubation in darkness that leads to intracellular energy depletion and triggers the release of stored sugars (Usadel et al., 2008), expression of SWEET17 is up-regulated in the root elongation zone (Fig. 2, J and L). The induced expression in dark suggests that SWEET17 also can function in Frc export from the vacuole to meet energy requirement *in planta*. The exporter activity of SWEET17 became evident under conditions in which high levels of Frc are accumulated in vacuoles, i.e. cold stress (Kaplan et al., 2004; Wormit et al., 2006). The reduced Frc accumulation by ectopic expression of SWEET17 demonstrated that SWEET17 can passively export Frc out of vacuoles along a concentration gradient *in vivo* as observed in a transport assay performed in a heterologous system (Chardon et al., 2013). Despite the distinct reduction of Frc content in leaves was observed (Fig. 5), we did not observe phenotypic differences between overexpressors and wildtype plants (data not shown). It is possible that other compensatory mechanisms such as organic compounds and sugar

1 alcohols are also induced during the osmotic adjustment to sustain cold tolerance
2 (Sanghera et al., 2011).

3 Despite the fact that SWEET17 protein levels of leaves were extremely low (Fig. 2F;
4 Supplemental Fig. S3A and S5), Frc content of leaves was dramatically increased in
5 leaves of *sweet17* mutants under normal conditions (Chardon et al., 2013). The reduced
6 Frc uptake activity of leaf-derived vacuoles from *sweet17-1/sweet16-1* demonstrates that
7 despite the low levels of protein, SWEET17 is likely function in leaf vacuoles
8 (Supplemental Fig. S12A). Alternatively, the reduced import activity of Frc in roots of
9 *sweet17* mutants may reduce their storage capacity and sink strength, which, in turn, can
10 reduce Suc unloading and ultimately increase sugar or carbohydrate accumulation in leaf
11 mesophyll cells (Ayre, 2011).

12 **SWEET17 acts as a bi-directional Frc-specific facilitator**

13 Transport assays using isolated leaf mesophyll vacuoles verified that SWEET17 is
14 an important vacuolar Frc-specific transporter *in vivo* (Fig. 5; Supplemental Fig. S12A).
15 Although we cannot exclude the possibility that SWEET17 exhibits a minor transport
16 activity for other hexoses or disaccharides, such activities would be very low compared to
17 Frc (Fig. 6B; Supplemental Fig. S8). Furthermore, excess Suc or Glc did not affect the
18 expression level of SWEET17-GUS (Supplemental Fig. S3B). When sugar accumulation
19 is induced by cold stress (Wingenter et al., 2010), only the concentration of Frc was
20 significantly affected in leaves of SWEET17 overexpressors (Fig. 5; Supplemental Fig.
21 S7B) and in vacuoles lacking SWEET17 (Chardon et al., 2013). Thus, unlike Arabidopsis
22 vacuolar AtTMTs (Schulz et al., 2011), AtVGT1 (Aluri and Buttner, 2007) and AtESL1
23 (Yamada et al., 2010) which may act as general tonoplastic hexose transporters (Wormit
24 et al., 2006), SWEET17 exhibits Frc specific transport functions *in vivo* and is probably
25 regulated by Frc-specific signaling. Moreover, insensitivity of the transport activity to
26 ATP and treatments with NH_4^+ and CCCP (Fig. 6B) clearly showed that SWEET17 acts
27 as a proton-independent bi-directional facilitator *in vivo*. This finding was consistent with
28 our functional assays (Fig. 4 and 5) and previous observations performed in a
29 heterologous oocyte system (Chardon et al., 2013). The uniport feature concurs with that
30 of other plasma membrane SWEET members (Chen et al., 2010; Chen et al., 2012). One

common property of all SWEETs described is the high apparent K_m value ranging from 9 mM for SWEET1 (to Glc) (Chen et al., 2010) to 71 mM for SWEET12 (to Suc) (Chen et al., 2012). Similarly, the high K_m value at 25.6 mM determined here for SWEET17 (Fig. 6C) suggests that vacuolar SWEETs share this property with those of the plasma membrane. An even higher K_m of 102 mM was reported for the vacuolar monosaccharide facilitator, AtESL1 (Yamada et al., 2010). A relative lower K_m for Frc transport compared to Glu may reflect the preference of many plants to accumulate more Glc than Frc.

Clade IV SWEETs function as vacuolar transporters

SWEET17 has a very close homolog, SWEET16 (Supplemental Fig. S9A), which has recently been demonstrated to function also as a vacuolar transporter (Klemens et al., 2013). Furthermore, using a promoter-GUS construct it had been shown that SWEET16 is mainly expressed at very low levels in xylem parenchyma cells in leaves and flower stalks (Klemens et al., 2013). However, similar to *SWEET17*, our expression analysis by (qRT)-PCR as well as by the GUS reporter showed that SWEET16 is predominantly expressed in roots at all stages (Fig. 1; Supplemental Fig. S1 and S10, A and B). Highest expression was detected in the cortex cells of mature roots (Supplemental Fig. S10, C and D). Although our expression profiles are different from the previous report (Klemens et al., 2013), all our results were well in line with data from public expression databases (Supplemental Fig. S1 and S2). Moreover, localization of a stable translational GFP fusion clearly assigned SWEET16 to the tonoplast in Arabidopsis roots (Supplemental Fig. S10, E-G), as suggested using the transient expression in leaf protoplasts (Klemens et al., 2013). Therefore, we conclude that SWEET16, like SWEET17, mainly functions in roots. Compared with other SWEETs, SWEET16 and SWEET17 are the only two SWEETs found to be highly expressed in roots (Fig. 7; Supplemental Fig. S9B). These results imply that the clade IV SWEETs have evolved from other SWEET clades to specifically function on root vacuoles. This is in analogy with the ALMTs or full-size ABC transporter family in which one clade (ALMT) and one subfamily (ABCCs) are also localized in the vacuolar membrane, while the others are localized in the plasma membrane (Kang et al., 2011).

From their similarities in amino acids, expression patterns, and localizations we expected that these two SWEETs would either function in a complementary way, e.g. one transporting Glc, the other Frc or redundantly. Indeed, mutations in both SWEET17 and SWEET16 dramatically reduced the Frc uptake activity of vacuoles to Frc *in vivo* (Supplemental Fig. S12A). A reduced accumulation of Frc was also observed in a SWEET16 overexpressor line under cold stress (Supplemental Fig. S7B). However, in contrast to the previous study performed in an oocyte expressing system (Klemens et al., 2013), we did not detect any transport activity of SWEET16 to sugars (Supplemental Fig. S12B; data not shown). It is possible that the uptake mediated by SWEET16 on the tonoplast was below the detection limit due to a high background (Fig. 6B; data not shown) (Klemens et al., 2013) than SWEET17 (K_m lower than 10 mM measured using an oocyte system) (Chardon et al., 2013). Nevertheless, similar to SWEET17, altered sensitivity of the root growth to excess Frc was observed in *sweet16* mutants and overexpressors (Fig. 8). However, a loss-of-function of both *sweet16* and *sweet17* double mutant did not further reduce the root tolerance to excess Frc compared to *sweet16* single mutant (Fig. 8A). These results indicate that SWEET17 and 16 may function in independent pathways by exchanging sugars across the tonoplast of root cells. Given that *SWEET17* is expressed to a much higher level in roots (Fig. 1; Supplemental Fig. S1), it is likely that SWEET17 is the dominant Frc transporter on the root tonoplast.

In summary, our work provides a functional characterization of the vacuolar Frc-specific uniporter SWEET17. Based on these results, we propose that when photosynthesis is very active, Suc is allocated from the leaf to the root where Suc is hydrolyzed to Glc and Frc that are imported into vacuoles for storage by hexose transporters and SWEET17, respectively. Depending on the metabolic stage of the root cells, defect of SWEET17 will either reduce the vacuolar loading or unloading. In this case, excess of Frc in the cytosol will lead to growth retardation due to toxic effects of Frc, while under energy-limiting conditions the plant is not able to use all the carbohydrate reserves. During these processes, SWEET16 may also contribute to sugar compartmentation across the root vacuole independently from SWEET17.

Materials and Methods

Plant and growth conditions

Arabidopsis plants and mutants used in this study are all in Columbia ecotype. Arabidopsis thaliana were grown with potting medium or solid agar media in a controlled growth room (22/18°C day/night temperature, 16-h light/ 8-h dark regime of $\sim 100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ illumination). For treatments of excess sugars, surface sterilized seeds (30% of concentrated bleach and 0.1% triton) were stratified at 4°C in the dark for 3 d and sown on 1/2MS solid media supplemented with 1% (w/v) Suc. After 5 d of growth, similar sizes of seedlings were transferred to 1/2MS media supplemented with various concentrations of Frc, Glc, or Suc as indicated. Expression patterns of reporter genes were observed after 2 d of transfer. To analyze effects on root growth, lengths of primary roots were recorded after 6 d of transfer and the relative root growth was determined using National Institutes of Health Image J 1.46v. For sugar content analysis, plants were grown in the growth chamber under 8-h light/16-h dark regime for 6 weeks. For treatment of cold stress, plants were grown for 5 weeks and subsequently transferred to a cooled growth chamber (4°C) for 1 week before analysis. For vacuole isolation, plants were grown for 4 to 5 weeks in a growth chamber under 8-h light/16-h dark regime, then transferred to low light condition ($40 \mu\text{mol m}^{-2} \text{ s}^{-1}$) for 5 d before isolation. For liquid culture, seedlings were grown for 7 d on a mesh with Murashige and Skoog (MS) solid media containing 1% Suc. Plants were then transferred to MS liquid media containing 1 to 2% Suc and cultured for 16 d before analysis. The MS or 1/2MS media used in this study included full or half strength of MS salt, respectively, 0.05 % (w/v) MES, and 1.5% agar for solid medium (adjust to pH 5.7 with KOH).

Generation of plants expressing GUS and GFP reporter genes

For $P_{SWEET17}$:GUS and $P_{SWEET16}$:GUS transcriptional constructs, *SWEET17* and *16* promoter fragments containing the 5' UTR region were amplified from Arabidopsis genomic DNA with Phusion polymerase (New England Biolabs, MA, USA) and specific primers (5-PSWT17-BP and 3-PSWT17-BP for $P_{SWEET17}$; 5-PSWT16-BP and 3-PSWT16-BP for $P_{SWEET16}$). The resulting 2746 bp and 1521 fragments, respectively, were first cloned into pDONR221-f1 and then transferred to a binary vector, pWUGW that contains a *GUS* reporter gene, using Gateway technology (Invitrogen, CA, USA). For

SWEET17-GUS/GFP and SWEET16-GUS/GFP translational constructs, the promoter fragments were first amplified from the pDONR221-f1 clones mentioned above and cloned into pGEM-T Easy (Promega, WI, USA). The promoter fragments were then cloned via *Pst*I and *Kpn*I sites into pMDC32-SWEET17 and pMDC32-SWEET16 constructs (refer to the section of “Overexpression of SWEET17 and 16 in Arabidopsis”) to replace the 35S promoter in those plasmids. The full P_{SWEET17}-SWEET17 and P_{SWEET16}-SWEET16 genomic fragments were then amplified using Phusion polymerase, cloned into pGEM-T Easy, digested, and subcloned into *Sac*II and *Pst*I sites of pUTKan or pGTkan, harboring the *GUS* or *GFP* reporter gene, respectively. All constructs were transformed into wildtype Arabidopsis plants using *Agrobacterium tumefaciens* strain C58 PGV3850 and flower dipping method (Clough and Bent, 1998). Transformants were identified on 1/2MS medium containing 50 µg mL⁻¹ Kanamycin. For analysis of reporter gene expression, plants from six independent transgenic lines were examined for each construct. Patterns of gene expression were consistent within a construct and representative 3 homozygous lines were further analyzed in other experiments. Sequences of primers were listed in Supplemental Table S1.

Histochemical localization of GUS

Expression of transcriptional or translational fusions of the GUS reporter gene was analyzed by histochemical staining. Transgenic plants or excised plant tissues were stained at 37°C for 2 h or 4 h as indicated using X-Gluc solution (0.1 M NaH₂PO₄, 10 mM ethylenediaminetetraacetic acid, 0.5 mM each of potassium ferricyanide and potassium ferrocyanide, 0.1% Triton X-100 and 0.25 mg mL⁻¹ X-glucuronidecyclohexylamine salt). After staining, tissues were cleared by replacing the staining solution with several changes of 70% and 90% ethanol as necessary.

Confocal microscopy for GFP observation

Fluorescence imaging of plants expressing SWEET17-GFP and SWEET16-GFP was performed on a Carl Zeiss LSM780 confocal microscope (Instrument Development Center, NCKU). Seedlings were incubated in 6 µM of FM4-64 (Invitrogen, CA, USA) for 5 min before imaging in order to visualize the plasma membrane. GFP was visualized by excitation with an argon laser at 488 nm and spectral detector set between 500 and 545

1 nm for the emission. The red fluorescence of FM4-64 was visualized by excitation with
2 an argon laser at 561 nm and spectral detector set between 566 and 585 nm for the
3 emission.

4 **Identification of SWEET17/16 insertion mutants**

5 The T-DNA insertion mutants for SWEET17 (*sweet17-1* and *sweet17-2*) (Chardon
6 et al., 2013) and SWEET16 (*sweet16-1* and *sweet16-2*) were obtained from the Salk
7 Institute Genomic Analysis Laboratory (<http://signal.salk.edu/cgi-bin/tdnaexpress>):
8 SM_3_15143, as *sweet16-1*; SM_3_30077, as *sweet16-2*. To confirm the homozygosity
9 of T-DNA insertion lines, gene-specific primers were used to examine wildtype alleles
10 (swt17-1-RP and swt17-1-LP for *sweet17-1*; swt17-2-RP and SWT17-5-UTR for
11 *sweet17-2*; swt16-1-RP and swt16-1-LP for *sweet16-1*; swt16-2-RP and swt16-2-LP for
12 *sweet16-2*). Locations of T-DNA insertions in *sweet16* mutants were confirmed by
13 sequencing PCR products amplified with gene-specific primers (swt16-1-RP and swt16-
14 2-RP) and the corresponding T-DNA left border primer (Spm32). The positions were
15 located at 32 and 945 bp downstream of the translational start codon, corresponding to
16 the forth and the first exon in the *sweet16-1* and *sweet16-2* mutant, respectively
17 (Supplemental Fig. S11A). To confirm the expression level, RT-PCR was performed with
18 specific primers (SWT17-5-UTR and swt17-1-LP for *SWEET17*; SWT16-5-UTR and
19 SWT16-3-UTR for *SWEET16*) to amplify the full-length of cDNA. Sequences of primers
20 were listed in Supplemental Table S1.

21 **Overexpression of SWEET17 and 16 in Arabidopsis**

22 To express *SWEET17* and *SWEET16* under the control of the 35S promoter, full
23 length *SWEET17/16* genomic sequences including all introns were amplified using
24 Phusion polymerase with gene specific primers (SWT17-5-UTR-BP and SWT17-3-UTR-
25 BP for *SWEET17*; SWT16-5-UTR-BP and SWT16-3-UTR-BP for *SWEET16*). The
26 resulting 2856 bp and 2119 bp fragments were cloned into pDONR221-f1 and then
27 transferred to a binary vector pMDC32 (Curtis and Grossniklaus, 2003) via Gateway
28 technology. The resulting plasmids, pMDC32-SWEET17, pMDC32-SWEET16, and the
29 corresponding empty vector, pMDC32 without the Gateway cassette, were transformed
30 into wildtype Arabidopsis plants using the *Agrobacterium tumefaciens* strain C58

PGV3850 and flower dipping method (Clough and Bent, 1998). Transformants were identified on 1/2MS medium containing 25 $\mu\text{g mL}^{-1}$ hygromycin B. Six single-copy T2 lines were obtained and three homozygous lines of highest expression were used in this study. Sequences of primers were listed in Supplemental Table S1.

qPCR and RT-PCR analysis

For aerial tissues in mature plants, total mRNA was isolated from leaves, stems, flowers and siliques of 7 to 8-week-old soil grown plants. For mRNA from young seedlings, shoots and roots of 2-week-old plants or 7-d-old whole seedlings grown on 1/2MS agar were harvested for extraction. To analyze expression in mature roots, total mRNA was isolated from roots of 23-d-old liquid cultured seedlings. Total mRNA was isolated using TRIsure (Bioline, London, UK) reagent or RNeasy mini kit (Qiagen, Hilden, Germany) as instructed by the manufacture. The resulting cDNA produced by MMLV (Qiagen, Hilden, Germany) was diluted and used as the template and subjected to 25 to 30 cycles of PCR reaction (94°C for 30 s, 55°C for 30 s, and 72°C for 40 s) using Taq DNA polymerase and a pair of gene-specific primers as indicated. Amplification of an Actin cDNA (Actin2, At3G18780) using Act2-F and Act2-R primers (Supplemental Table S1) was used to normalize results from different samples. For RT-PCR, samples were separated on a 1.5% agarose gel. For real-time qPCR, the amplification was performed using HotStart-IT SYBR Green qPCR Master Mix (USB, OH, USA) or KAPA SYBR FAST qPCR Kit (Kapa Biosystems, MA, USA) according to the manufacturer's instructions on a 7300 PCR system (Applied Biosystems, CA, USA). The relative expression level was determined by comparing with the expression of Actin 2 ($1000 \times 2^{-\Delta(\text{Ct}_{\text{SWEET}} - \text{Ct}_{\text{Actin2}})}$). The gene-specific primers used in qRT-PCR for 17 Arabidopsis SWEET genes were listed in Supplemental Table S1.

Extraction and assay of soluble sugars

Ground, freeze-dried Arabidopsis rosette leaf material (~ 0.5 g FW) was extracted in 800 μl ice-cold 0.7 M perchloric acid for 5 min with intermittent mixing, using a Mixer Mill (Retsch, Haan, Germany). All the subsequent steps were carried out between 0 and 4°C. After centrifugation (5 min, 5000 g, 4°C), 600 μl of supernatant (soluble fraction) was adjust to pH 5 to 6 by adding 2 M KOH, 0.4 M 2-ethanesulfonic acid

(MES), 0.4 M KCl (approximately 0.4 ml per ml of supernatant). Precipitated potassium perchlorate was removed by centrifugation (10 min, 10000g, 4°C). For desalting, samples of the neutralized soluble fraction (250 µl) were applied to sequential 1.5-mL columns of Dowex 50 W and Dowex 1 (Sigma-Aldrich). The neutral compounds were eluted with 5 ml of water, lyophilized, and redissolved in 200 µl of water. Soluble sugars (Glc, Suc and Frc) were separated using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on a Dionex PA-20 column (Dionex, Thermo Scientific, MA, USA), according to the following conditions: eluent A, 100 mM NaOH; eluent B, 150 mM NaOH and 500 mM sodium acetate. At a flow rate of 0.5 mL min⁻¹, the gradient was as follows: 0 to 7 min, 100% A and 0% B; 7 to 26.5 min, a concave gradient to 20% A and 80% B (malto-oligosaccharide elution); 26.5 to 32 min, hold at 20% A and 80% B (column wash step); 32 to 40 min, step to 100% A and 0% B (column re-equilibration). Peaks were identified by co-elution with known malto-oligosaccharide standards. Peak areas were determined using Chromeleon software (Dionex, Thermo Scientific, MA, USA).

Vacuole isolation from Arabidopsis leaves

Vacuoles were isolated as described by Song et al. (2010) with some minor modifications. Firstly, to isolate mesophyll protoplasts, the abaxial epidermis of mature healthy leaves was abraded using sandpaper (p 500). The abraded leaves were floated on medium A (0.5 M Sorbitol, 1 mM CaCl₂ 2H₂O, 10 mM MES-KOH, pH 5.6) containing 1% Cellulase R10 and 0.5% Mazerozyme R10 for 1.5 h at 30°C. The released protoplasts were collected by centrifugation for 8 min at 400 g (4°C) on a cushion of osmotically stabilized Percoll (100% Percoll, 0.5 M Sorbitol, 1 mM CaCl₂ 2H₂O, 10 mM MES, pH 6). The supernatant was aspirated and the sedimented protoplasts were resuspended in the residual solution, which was completed to a final Percoll concentration of 40% with the osmotically stabilized Percoll. A Percoll gradient was formed by overlaying the suspended protoplasts with 3:7 (v/v) mix of Percoll pH 7.2 (500 mM sorbitol, 20 mM HEPES in Percoll 100%) and medium B (400 mM sorbitol, 30 mM potassium gluconate, 20 mM HEPES, pH 7.2 adjusted with imidazole), overlaid with medium B containing 1 mg mL⁻¹ BSA and 1 mM DTT. After centrifugation for 8 min at 250g (4°C), the

1 protoplasts were recovered from the upper interphase and lysed with the same of volume
2 of pre-warmed (42°C) lysismedium (200 mM Sorbitol, 20 mM EDTA, 10 mM HEPES
3 pH 8.0 with KOH, 10% Ficoll, 0.2 mg mL⁻¹ BSA, 1 mM DTT) under 37°C for
4 maximum 10 min. Vacuoles were purified by overlaying 5 ml of the lysate with 5 ml of
5 1:1 fresh lysismedium/medium C (400 mM betaine, 30 mM potassium gluconate, 20 mM
6 HEPES, pH 7.2 adjusted with imidazole, 1 mg mL⁻¹ BSA, 1 mM DTT) and 1 ml medium
7 C. After centrifugation for 8 min at 1300g (4°C), the vacuoles were recovered from the
8 interface between the middle and upper layer. Microscopic analysis indicated that
9 contamination with intact protoplasts was less than 3%.

10 **Transport analysis with Arabidopsis vacuole**

11 Transport experiments were performed using silicone oil centrifugation technique as
12 described previously (Song et al., 2010). The Michaelis-Menten nonlinear least-square
13 regression fits were calculated using the SSmicmen function without initial parameters
14 within the nls function of R 2.14.0 (www.R-project.org).

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1 **SUPPLEMENTAL MATERIALS**

2 **Supplemental Table S1. Specific primers used in this study.**

3 **Supplemental Figure S1. Organ-specific expression of Arabidopsis *SWEET17* and**
4 ***16*.**

5 **Supplemental Figure S2. Cell-type specific expression of Arabidopsis *SWEET17* and**
6 ***16* genes in roots.**

7 **Supplemental Figure S3. Developmental expression of *SWEET17* and its regulation**
8 **in response to the sugar status.**

9 **Supplemental Figure S4. Expression of *SWEET17*-GUS fusion proteins in shoots**
10 **under normal conditions or in response to darkness and cold.**

11 **Supplemental Figure S5. Tonoplast localization of *SWEET17*-GFP fusion proteins**
12 **in leaves.**

13 **Supplemental Figure S6. Functional characterization of *SWEET17* overexpressing**
14 **lines.**

15 **Supplemental Figure S7. The sugar composition in leaves of *SWEET17* and *16***
16 **overexpressors.**

17 **Supplemental Figure S8. Analysis of sugar transport activity of vacuoles ectopically**
18 **expressing Arabidopsis *SWEET17*.**

19 **Supplemental Figure S9. Comparison of Arabidopsis clade IV *SWEETs*.**

20 **Supplemental Figure S10. Expression patterns of *SWEET16*-GUS/GFP fusion**
21 **proteins *in planta*.**

22 **Supplemental Figure S11. Identification of *SWEET16* mutants and overexpressing**
23 **lines.**

1 **Supplemental Figure S12. Characterization of SWEET16 transport activity *in***
2 ***planta*.**

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1 **Figure Legends**

2 **Figure 1.** Developmental expression profile of the SWEET17 gene in Arabidopsis. Total
3 RNA was isolated from roots (R) and shoots (S) of 2-week-old seedlings or new leaves
4 (NL), mature leaves (ML), stems (St), flowers (FL), siliques (Si) of 7 to 8-week-old soil-
5 grown plants. The resulted cDNA was used for qRT-PCR with specific primers for
6 SWEET17 and 16. The y axis indicates the relative expression level normalized to an
7 internal control Actin2. The results are means \pm SE from three independent biological
8 repeats. Figure 1. Developmental expression profile of the SWEET17 gene in
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14 independent biological repeats.

15 **Figure 2.** Expression patterns of SWEET17 in Arabidopsis. Histochemical analysis of
16 GUS activity in transgenic Arabidopsis expressing SWEET17-GUS fusion proteins
17 driven by the *SWEET17* native promoter. A, Seven-d-old seedlings. B, Two-week-old
18 plate grown plants. C and D, Root tip and mature zone of 7-d-old seedlings. E, Hand
19 section of (D). F, Mature rosette leaf. G, Flower. H, Silique. I, Seed. Effects of Frc and
20 darkness on the expression of SWEET17-GUS are also demonstrated. Five-d-old
21 seedlings were treated with no sugar (J), 1% Frc (K) and no sugar in the dark (L) for 2 d
22 and then the histochemical staining was performed. The reaction time for GUS staining is
23 2 h for seedlings and all root tissues (A-E; J-L) and 4 h for leaves, flowers, siliques and
24 seeds (F-I). Bars = 1 mm in A, B, F and G; 0.5 mm in H; 100 μ m in C-E and I-L.

25 **Figure 3.** Tonoplast localization of the SWEET17-GFP fusion protein in Arabidopsis
26 roots. Arabidopsis plants were stably transformed with a SWEET17-GFP construct under
27 the control of the SWEET17 native promoter. Fluorescence localizations of GFP fusion
28 proteins in 7-d-old seedlings were imaged by confocal microscopy. A-C, root tips. D-F,
29 mature roots. The GFP fluorescence images (A)(D), the FM4-64 staining (6 μ M for 5

min) to label the plasma membrane (B)(E), and the merged images (C)(F) of the same cells are shown. The arrowheads point to the SWEET17-GFP labeled vacuole membranes lining around the side of the nucleus. Bar = 10 μ m.

Figure 4. Altered expression of *SWEET17* affects sensitivity of root growth to excess Frc. Arabidopsis seeds were germinated and grown on media supplemented with 1% sucrose for 5 d, then seedlings were transferred to media containing various concentrations of Frc. Relative growth of primary roots was measured after 6 d of treatments. A, Comparison of root growth in two independent *sweet17* mutant lines and the wildtype that was identified from the segregating mutant population (Col-TDNA). B, Comparison of root growth in three independent *SWEET17* overexpressor lines (35S:*SWEET17*-1, -6 and -2) and the wildtype that was transformed with the empty vector (Col-Vector-1 and -2). The data presented are means \pm SE (n = 8). Significant differences from the wildtype were determined by Student's t test indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

Figure 5. Ectopic expression of *SWEET17* reduces Frc accumulation in leaves under cold stress conditions. Sugar contents in leaves of transgenic Arabidopsis expressing a 35S:*SWEET17* transgene were shown. Samples were harvested from 6-week-old soil-grown plants treated with cold stress (4°C) for 1 week. Results are means \pm SE (n = 9). Significant differences from the wildtype transformed with the empty vector (Col-Vector) or identified from the segregating mutant population (Col-TDNA) were determined by Student's t test indicated by asterisks: ** $P < 0.01$.

Figure 6. The transport activity of Arabidopsis SWEET17 *in vivo*. A, Time course of Frc uptake into mesophyll vacuoles isoated from plants expressing the empty vector (Col-Vector) or the 35S:*SWEET17* transgene. Vacuoles were incubated with 0.2 mM of 14 C-labeled Frc. Results are means \pm SE (n = 3 to 4). Significant differences from vacuoles expressing the vector were determined. B, Effector analysis of Frc uptake into vacuoles isolated from plants expressing the empty vector or the 35:*SWEET17* transgene. The uptake level under treatments of no ATP, 5 mM NH_4Cl and 10 mM CCCP was determined. Results are means \pm SE (n = 3 to 4). Significant differences from vacuoles expressing the 35:*SWEET17* and incubated with 4 mM ATP were determined. C, Kinetic

1 analysis of Frc uptake activity in vacuoles isolated from plants overexpressing *SWEET17*.
2 Results are means \pm SD (n = 4). In B-C, the uptake level was determined after 3 to 20
3 min of incubation in 0.2 mM (B) or indicated (C) 14 C-labeled Frc. Significant differences
4 were determined by Student's t test indicated by asterisks: ** $P < 0.01$.

5 **Figure 7.** Expression of Arabidopsis *SWEET* genes in roots. Total RNA was isolated
6 from roots of 23-d-old liquid cultured seedlings and the cDNA was used for (qRT)-PCR
7 with specific primers for each *SWEET* gene. The y axis indicates the relative expression
8 level normalized to internal control *Actin2*. The results are means \pm SE from four
9 independent biological repeats.

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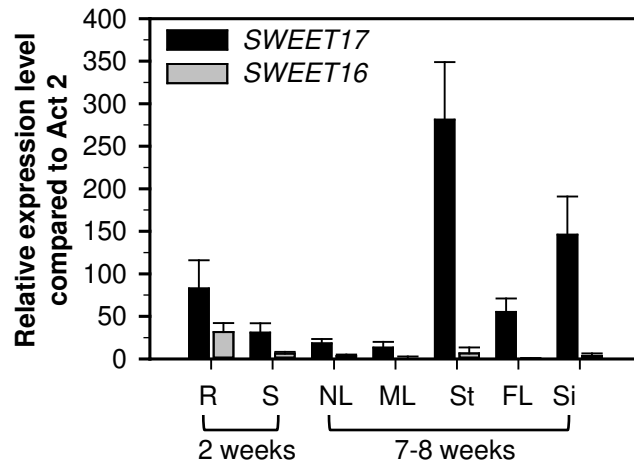


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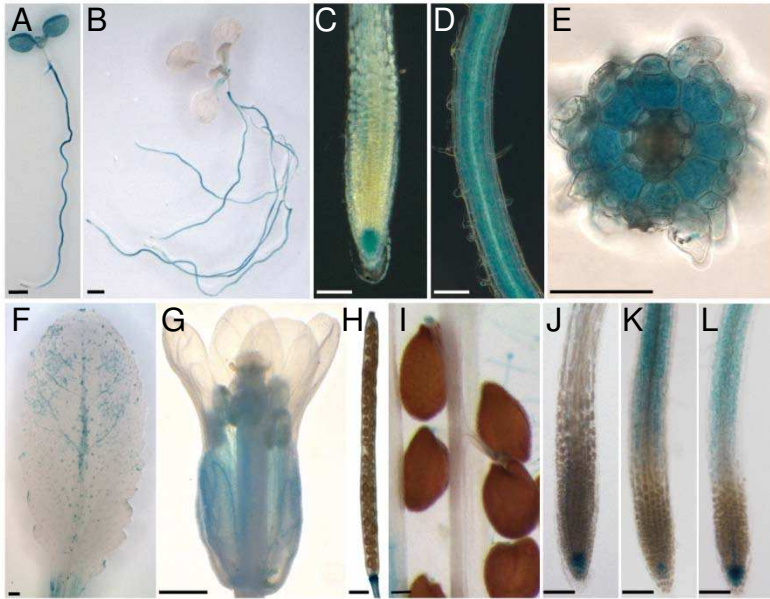


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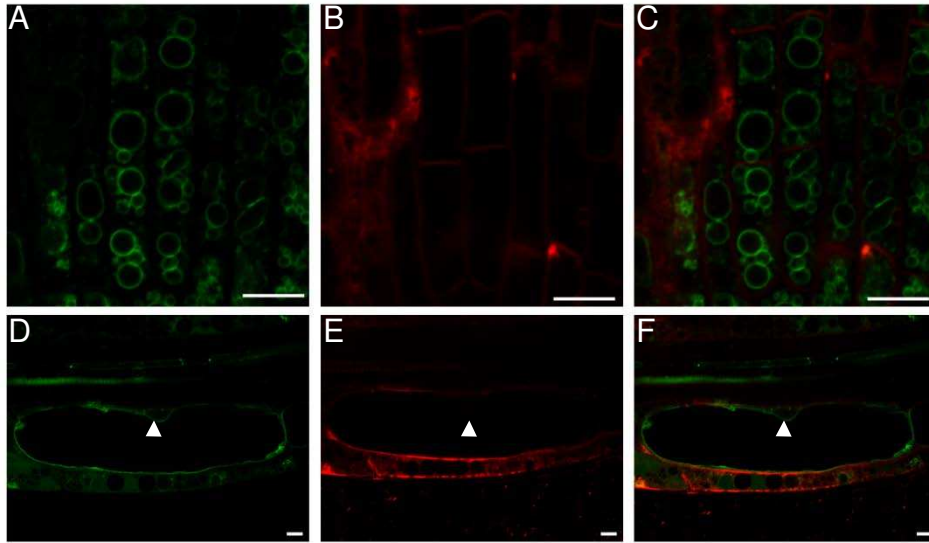


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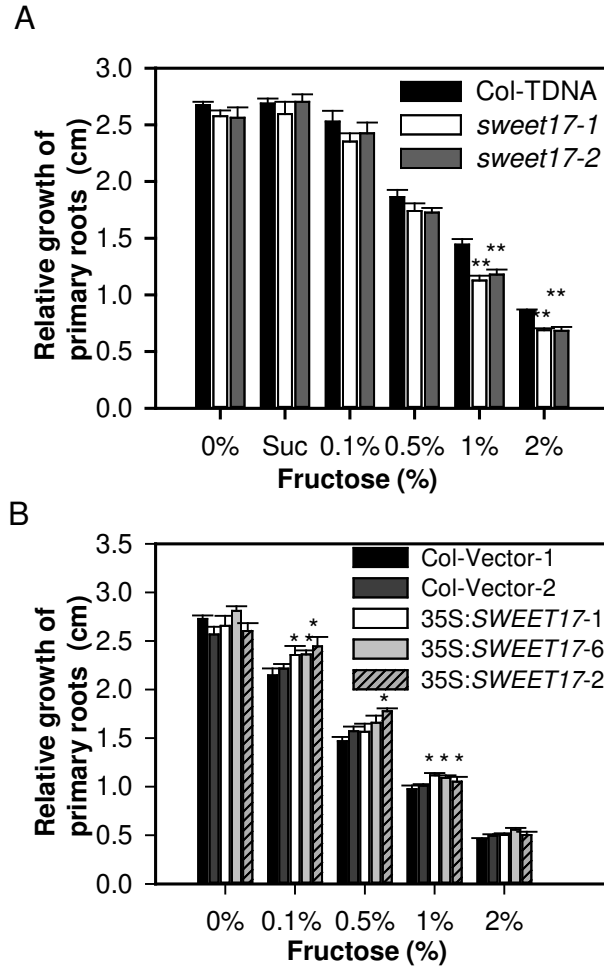


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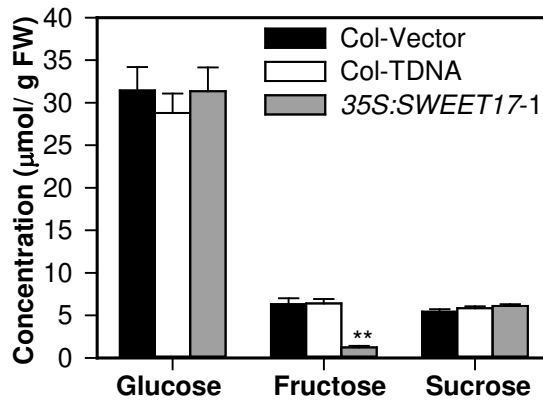


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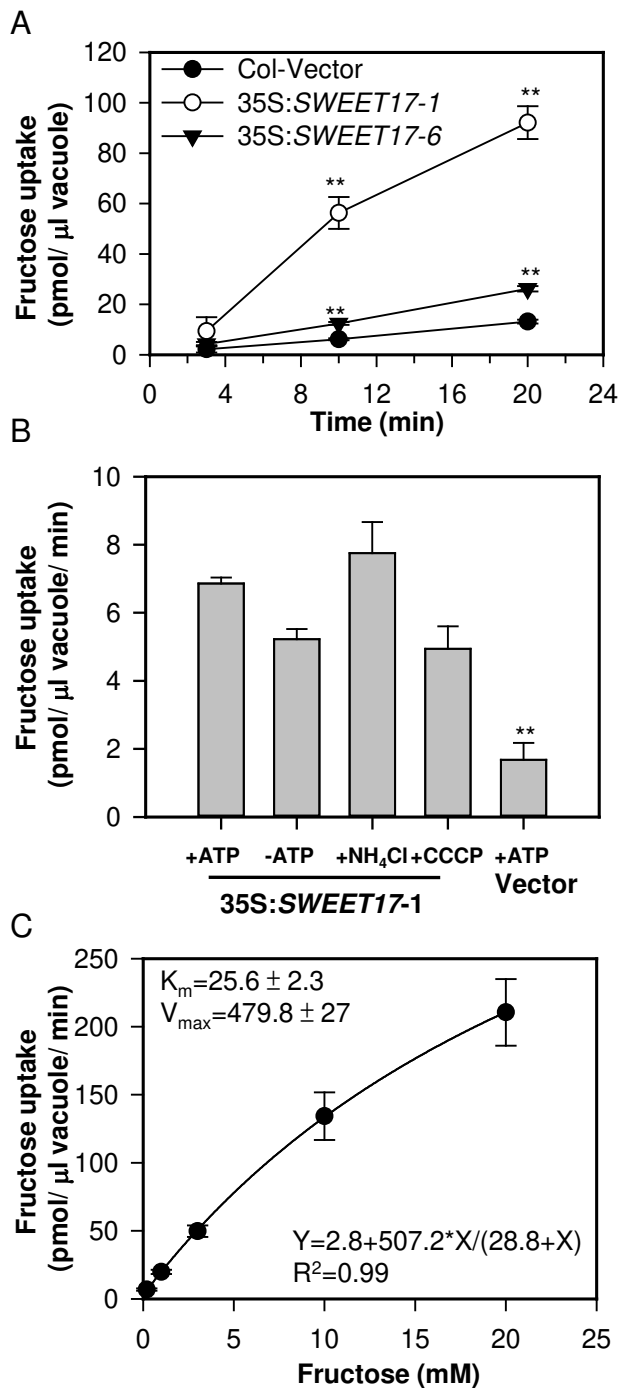


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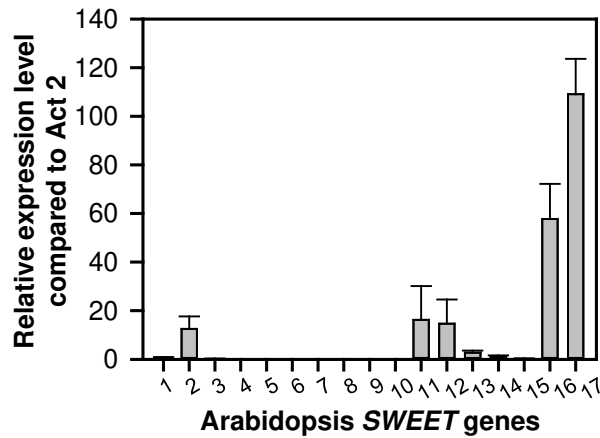


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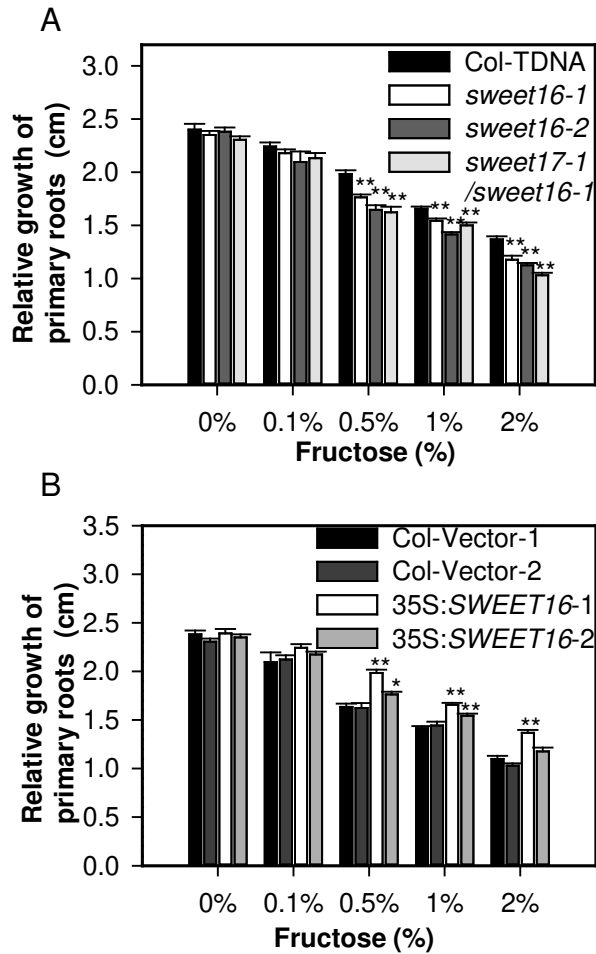
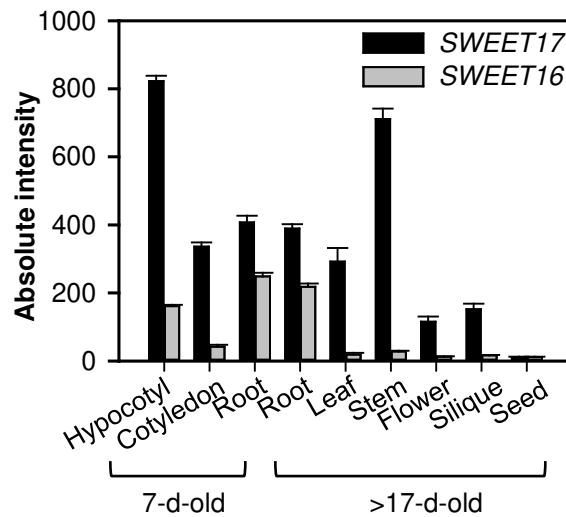
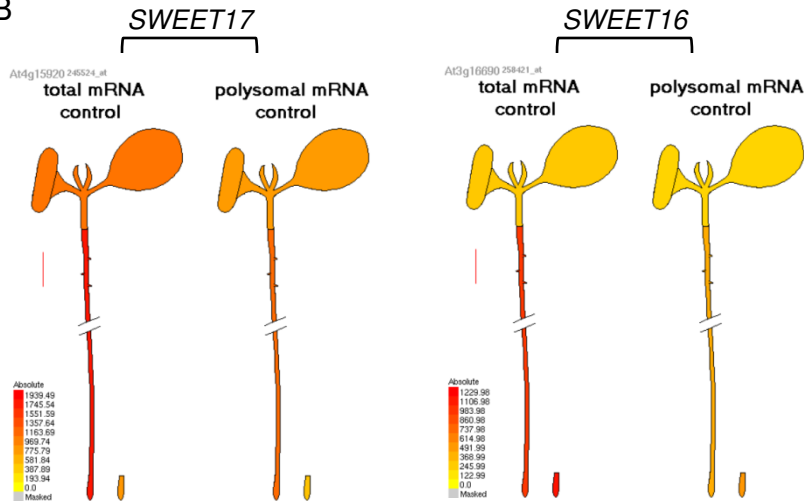


Figure 8. Altered expression of *SWEET16* affects sensitivity of root growth to excess Frc. Arabidopsis seeds were germinated and grown on media supplemented with 1% Suc for 5 d, then seedlings were transferred to media containing various concentrations of Frc. Relative growth of primary roots were measured after 6 d of treatments. A, Comparison of root growth in two independent *sweet16* mutant lines and the wildtype that was identified from the segregating mutant population (Col-TDNA). B, Comparison of root growth in two independent *SWEET16* overexpressor lines (35S:*SWEET16-2*, -3) and the wildtype that was transformed with the empty vector (Col-Vector-1, -2). The data presented are means \pm SE (n = 8). Significant differences from the wildtype were determined by Student's t test indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

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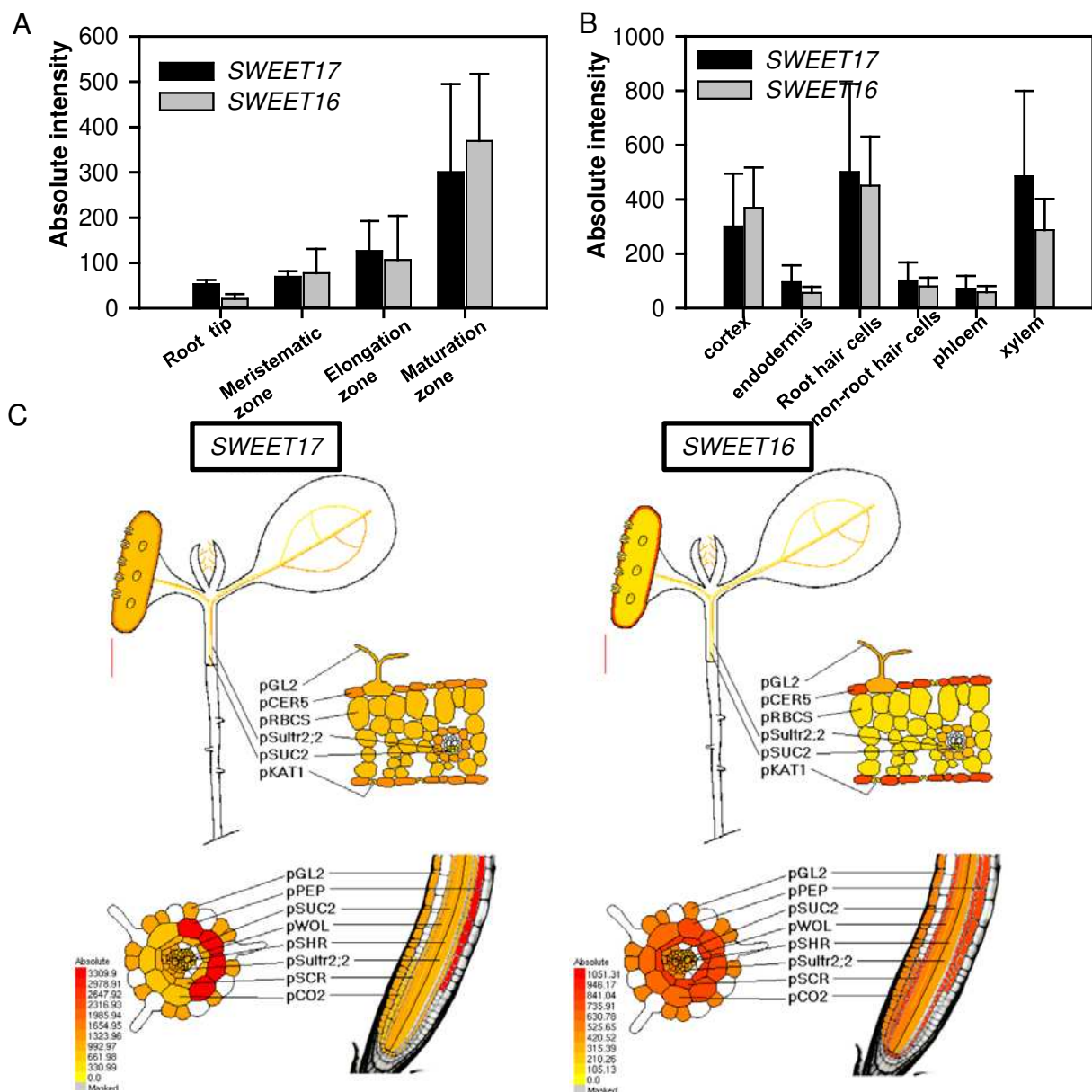


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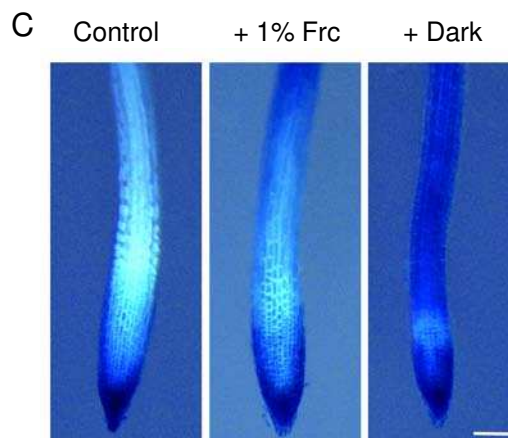
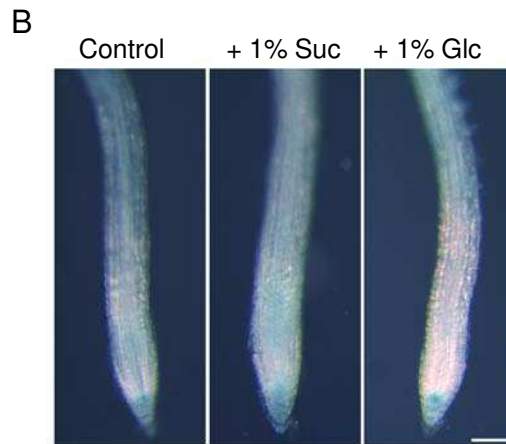
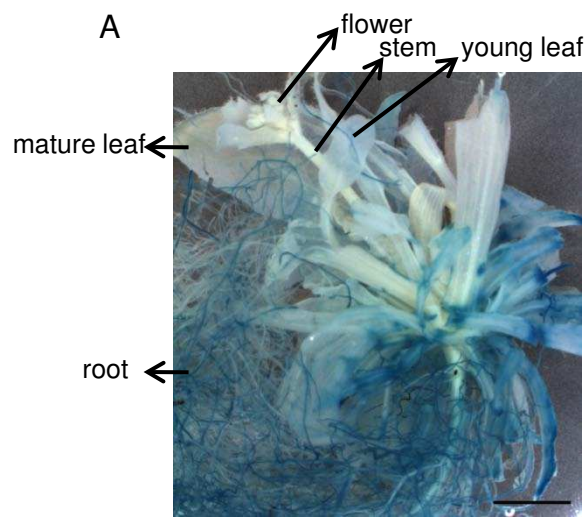
Supplemental Figure S1. Organ-specific expression of Arabidopsis *SWEET17* and *16*.

(A) Transcriptional levels of *SWEET17* and *16* genes in different developmental stages deduced from the Arabidopsis eFP Browser (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). Results are means \pm SD from three replicates. (B) Displays of absolute expression values of polysomal mRNA in roots and shoots obtained from the Arabidopsis Translatome eFP Browser (<http://efp.ucr.edu/>). (C) Histochemical staining of 7-d-old Arabidopsis seedlings expressing the *SWEET17* promoter:GUS transgene was shown.



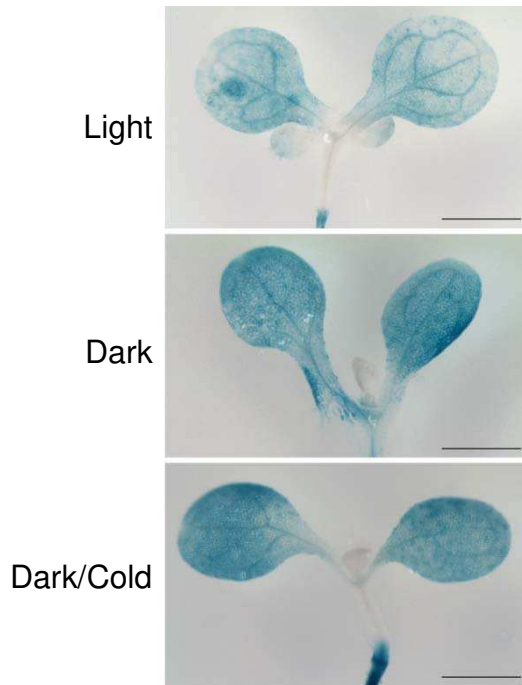
Supplemental Figure S2. Cell-type specific expression of Arabidopsis *SWEET17* and *16* genes in roots.

(A) Transcriptional levels of *SWEET17* and *16* genes in different longitudinal sections of root cortex cells deduced from the Arabidopsis eFP Browser (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). Results are means \pm SD from two to three longitudinal sections of the same developmental stages. (B) Transcriptional levels of *SWEET17* and *16* in different tissues of mature roots deduced from the Arabidopsis eFP Browser. Results are means \pm SD from four longitudinal sections of the same tissues. (C) Display of absolute expression values of polysomal mRNA in specific cell types of roots and shoots obtained from the Arabidopsis Translatome eFP Browser (<http://efp.ucr.edu/>). Absolute expression levels of individual genes are shown by the corresponding color scales 2 shown in each panel.



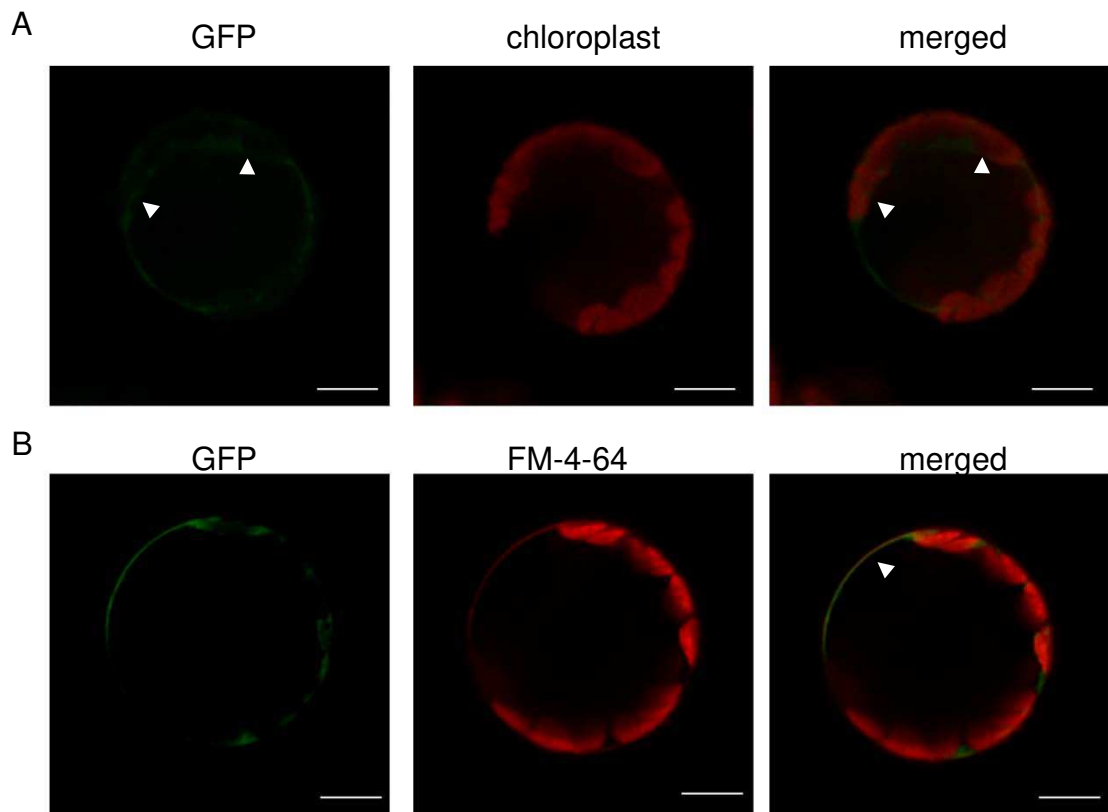
Supplemental Figure S3. Developmental expression of SWEET17 and its regulation in response to the sugar status.

(A) Histochemical staining of 8-week-old Arabidopsis plants expressing SWEET17-GUS fusions under the control of the *SWEET17* promoter was shown. (B) Effects of 1% Suc or Glc on the expression of SWEET17-GUS fusion proteins in root tips were analyzed in 7-d-old seedlings. (C) Expression of the GUS transgene driven by the *SWEET17* promoter was observed in 7-d-old Arabidopsis seedlings grown under control conditions or exposed to media containing 1% Frc or in the dark for 2 d. Bars = 5 mm in A and 100 μ m in B and C.



Supplemental Figure S4. Expression of SWEET17-GUS fusion proteins in shoots. under normal conditions or in response to darkness and cold.

Histochemical staining of 9-d-old *Arabidopsis* seedlings expressing SWEET17-GUS fusions under the control of *SWEET17* promoter was shown. Seedlings were grown under the control condition supplemented with light or exposed to darkness or combination of darkness and cold stress (4°C) for 2 d. Bar = 1 mm.



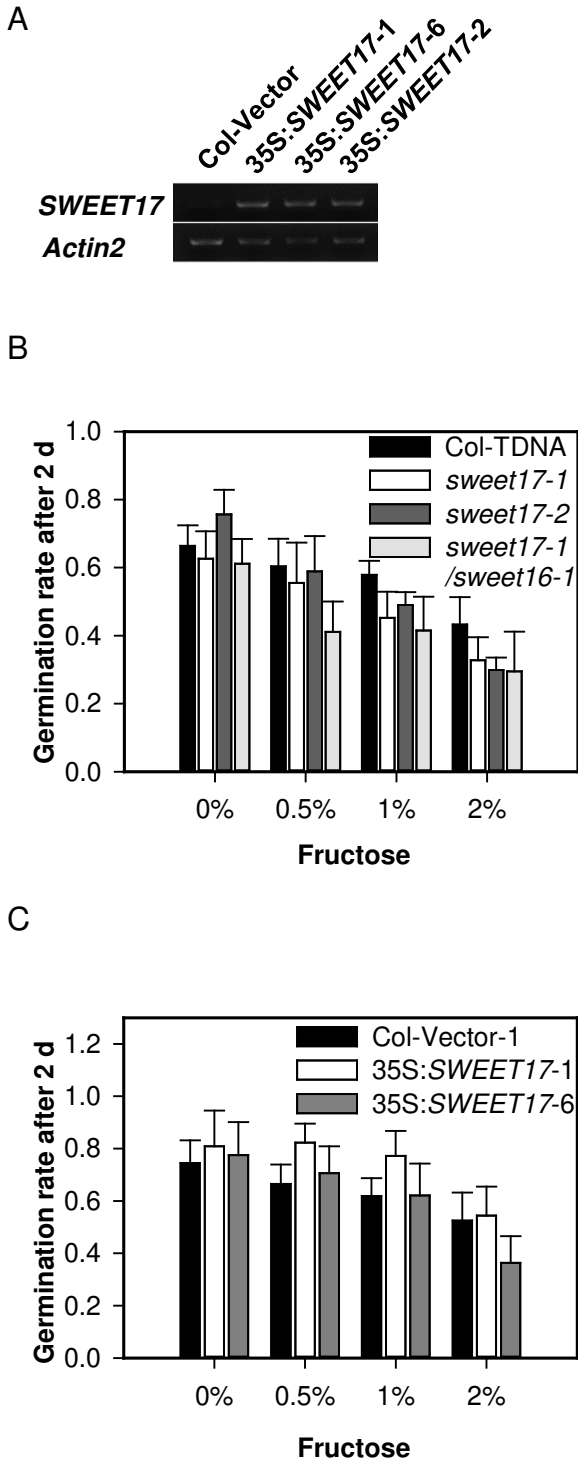
Supplemental Figure S5. Tonoplast localization of SWEET17-GFP fusion proteins in leaves.

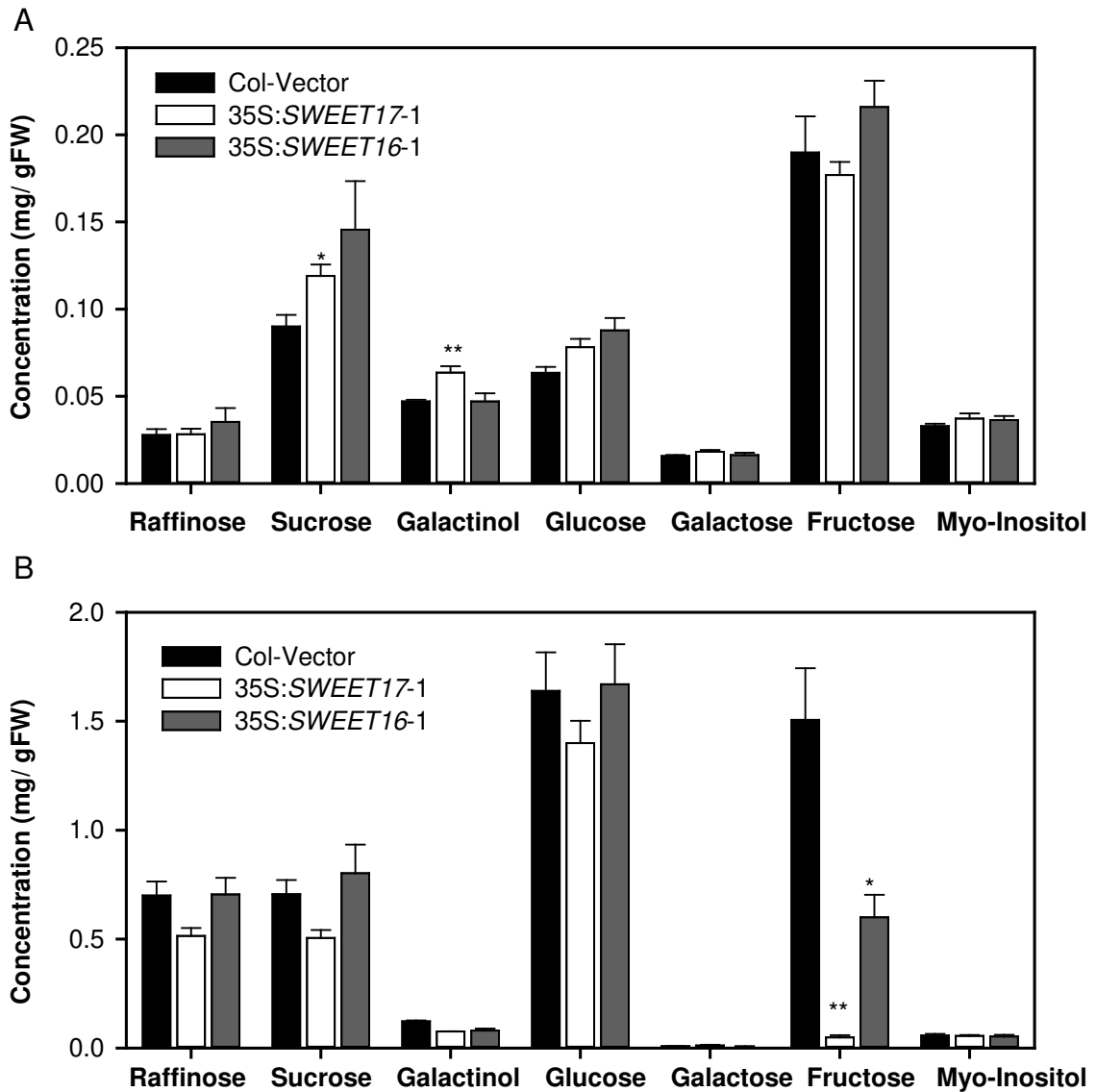
Arabidopsis plants were stably transformed with a SWEET17-GFP construct under the control of the *SWEET17* native promoter. The fluorescence of SWEET17-GFP fusion proteins in mesophyll protoplasts isolated from leaves of 2-week-old seedlings was shown without (A) or with FM4-64 staining (B). The individual optical sections of the same cells were imaged by a confocal microscopy. The arrowheads point to the SWEET16-GFP labeled vacuole membranes lining inside of chloroplasts (A) or the plasma membrane (B). Bar = 10 μm.

Supplemental Figure S6. Functional characterization of *SWEET17* overexpressing lines.

(A) RT-PCR analysis of *SWEET17* gene expression in transgenic Arabidopsis expressing the empty vector (Col-Vector) or the 35S:*SWEET17* transgene. Total RNA was isolated from 7-d-old seedlings and the resulted cDNA products were used for amplification with primers for *SWEET17*. Expression of *Actin2* was used as a loading control. Numbers indicates various independent transgenic lines.

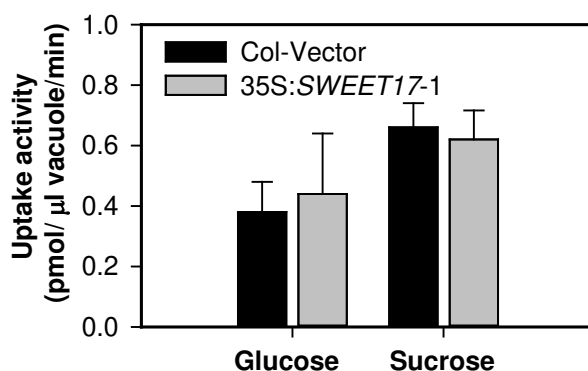
(B)(C) Germination efficiency under excess Frc. Seeds were germinated on media supplemented with various concentrations of Frc. Germination rates were calculated after 2 d between two independent *sweet17* mutants, the double mutant *sweet17-1/sweet16-1* and the wildtype that was identified from the segregating mutant population (Col-TDNA). The same experiment was also performed using two independent *SWEET17* overexpressors (35S:*SWEET17*-1 and -6) and the wildtype that was transformed with the empty vector (Col-Vector-1). The data presented are means \pm SE of four independent experiments. Significant differences from the wild type were determined by Student's t test.





Supplemental Figure S7. The sugar composition in leaves of *SWEET17* and *16* overexpressors.

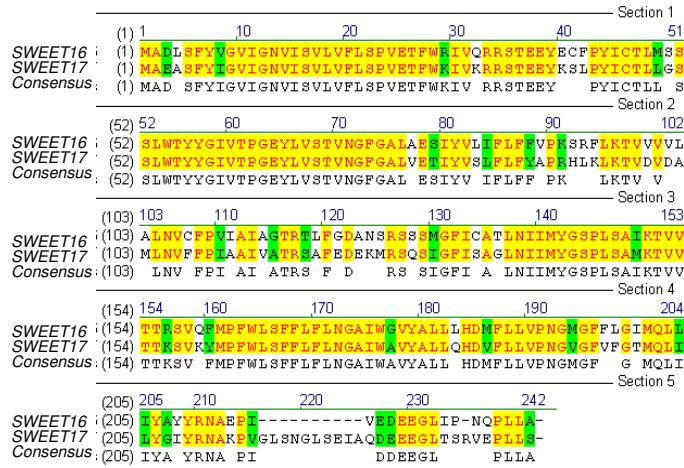
Leaves of transgenic *Arabidopsis* expressing the empty vector (Col-Vector), the 35S:*SWEET17* or 35S:*SWEET16* transgenes were harvested from 6-week-old soil-grown plants grown under normal conditions (A) or subjected to 1 week of cold stress (4°C) (B). Results are means \pm SE (n = 4). Significant differences from the wildtype (Col-Vector) were determined by Student's t test indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.



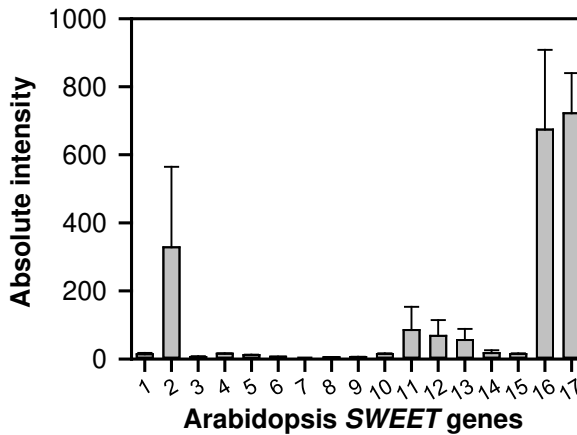
Supplemental Figure S8. Analysis of sugar transport activity of vacuoles ectopically expressing Arabidopsis *SWEET17*.

The uptake activity of Glc and Suc into vacuoles expressing the empty vector or the 35S:*SWEET17* transgene was determined by deducing the 3 min value from the 20 min to correct for vacuolar contamination with the medium containing 0.2 mM of ^{14}C -labeled Glu or Suc. Results are means \pm SE from three independent experiments.

A

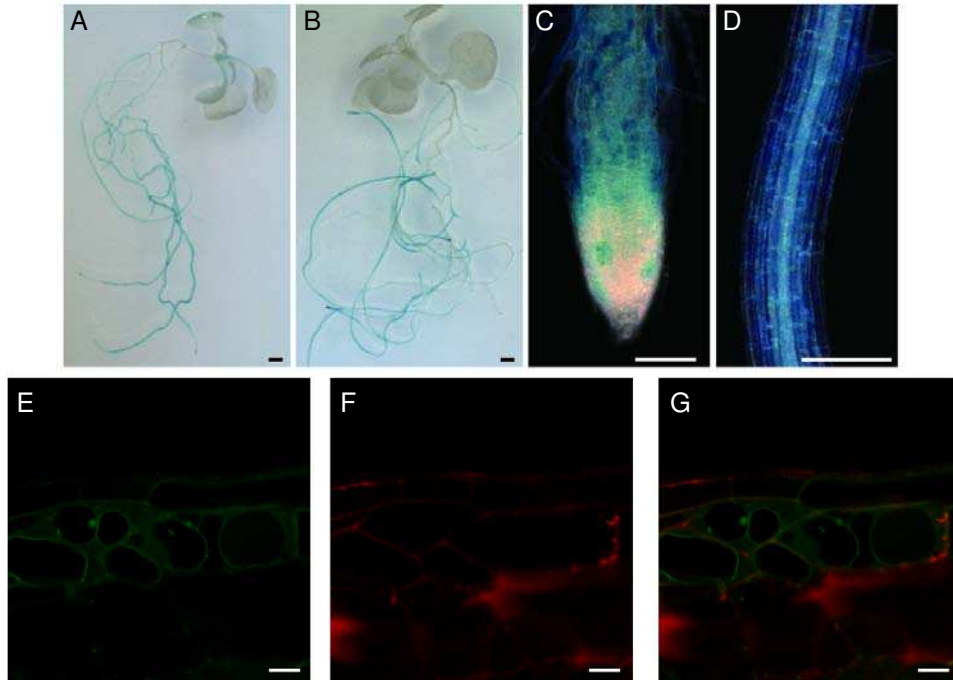


B



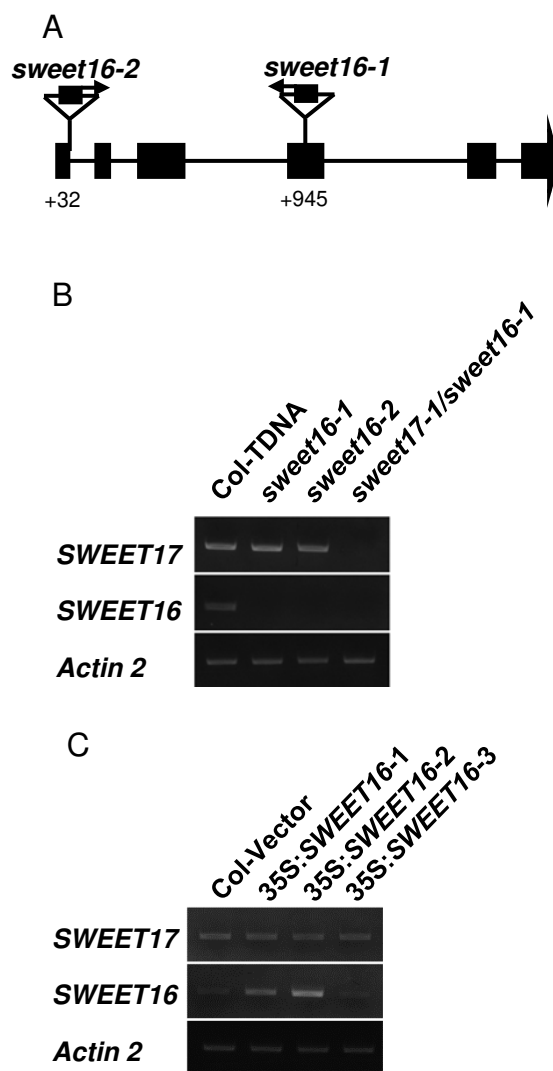
Supplemental Figure S9. Comparison of Arabidopsis clade IV *SWEET*s.

(A) The alignment of amino acid sequences of SWEET17 and 16 was shown. Regions marked in yellow and green indicate the identical and similar regions, respectively. (B) The expression profile of the Arabidopsis *SWEET* gene family in roots was deduced from AtGeneExpress database (<http://jsp.weigelworld.org/expviz/expviz.jsp>). Results are means \pm SD from different experiments.



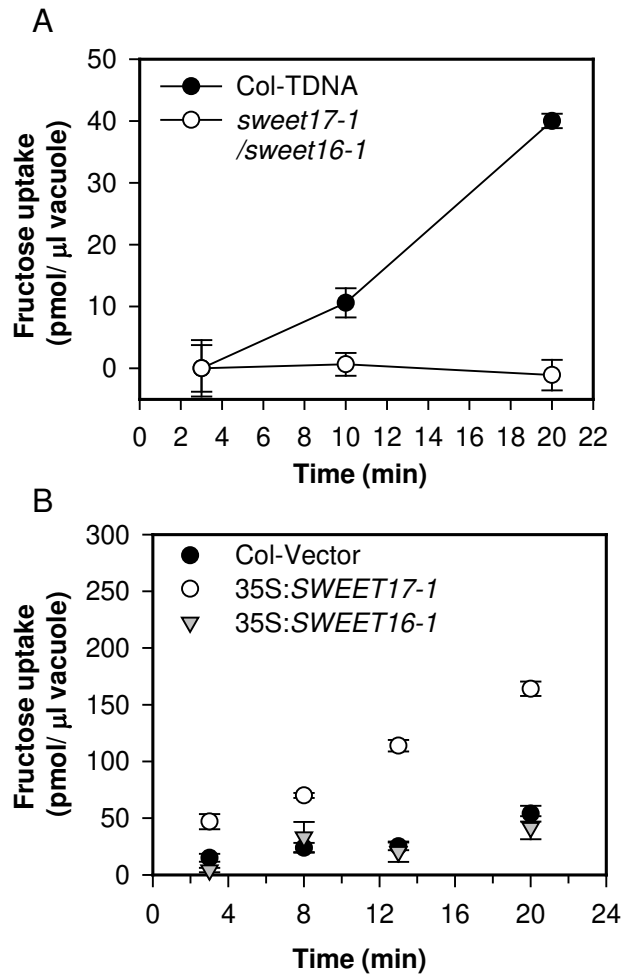
Supplemental Figure S10. Expression patterns of SWEET16-GUS/GFP fusion proteins *in planta*.

Histochemical analysis of GUS activity in 2-week-old transgenic *Arabidopsis* seedlings expressing the SWEET16-GUS fusion (A) or the transcriptional fusion (B) of *SWEET16* that were driven by the *SWEET16* native promoter. Images of stained root tips (C) and mature roots (D) of 7-d-old seedlings expressing SWEET16-GUS fusion proteins were shown. The fluorescence of SWEET16-GFP fusion proteins driven by the *SWEET16* native promoter were demonstrated in root tips (E) of 7-d-old seedlings imaged by a confocal microscopy. The red fluorescence derived from the FM4-64 staining indicated the localization of the plasma membrane (F) and the corresponding merged image in the same cell was shown (G). Bars = 1 mm in A, B and 100 μ m in C, D and 10 μ m in E-G.



Supplemental Figure S11. Identification of *SWEET16* mutants and overexpressing lines.

(A) The position and orientation of T-DNA insertions in *SWEET16*. Black boxes represent exon sequences and numbers indicate positions of insertions relative to the translational start codon. The arrows indicate the orientation of the left border. (B) RT-PCR analysis of *SWEET17* and *16* gene expression in single *sweet16* mutants, the *sweet17-1/sweet16-1* double mutant and the wildtype identified from the *sweet16-1* segregating population (Col-TDNA). (C) RT-PCR analysis of *SWEET17* and *16* gene expression in transgenic Arabidopsis expressing the empty vector (Col-Vector) or the 35S:*SWEET16* transgene. Numbers indicates various independent transgenic lines. In (B) and (C), total mRNA was isolated from 7-d-old seedlings and the resulted cDNA products were used for amplification with primers for *SWEET17* and *16* as indicated. Expression of *Actin 2* was used as a loading control.



Supplemental Figure S12. Characterization of SWEET16 transport activity *in planta*.

Time course of Frc uptake into vacuoles of the *sweet17-1/sweet16-1* double mutant and wildtype, Col-TDNA, isolated from the segregation mutant population (A), or those expressing the empty vector or the 35S:*SWEET17/16* transgenes (B). Vacuoles from leaf mesophyll cells were isolated from plants grown in soil for 4 to 5 weeks and incubated in 0.2 mM of ^{14}C -labeled Frc. Results are means \pm SE (n = 4).